PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/55, 15/32, 9/22, 15/00, 1/21, 15/70, 15/62, C12S 1/00, A61K 39/02, A01N 63/00 // C07K 14/325, (C12N 1/21, C12R 1:19)

A1

(11) International Publication Number: **WO 95/10614**

(43) International Publication Date: 20 April 1995 (20.04.95)

(21) International Application Number: PCT/DK94/00381

(22) International Filing Date: 12 October 1994 (12.10.94)

(30) Priority Data:
08/135,665 13 October 1993 (13.10.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/135,665 (CIP)
Filed on 13 October 1993 (13.10.93)

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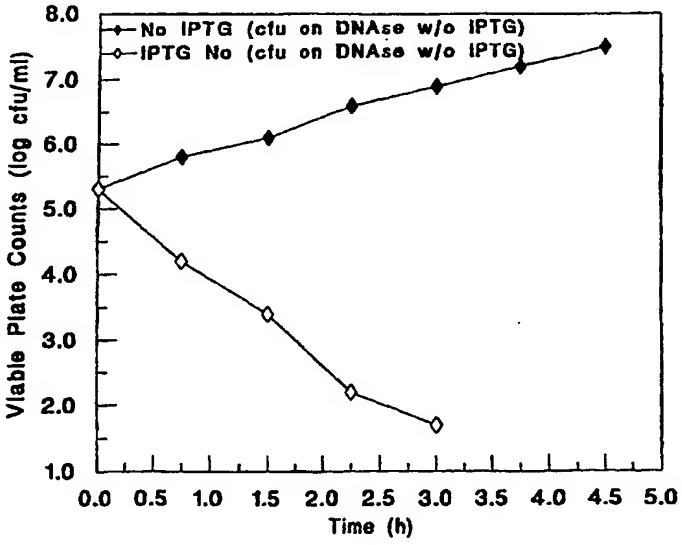
(81) Designated States: AM, AT, AT (Utility model), AU, BB, BG, BR, BY, CA, CN, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, FI, FI (Utility model), GE, HU, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, NO, NZ, PL, RO, RU, SI, SK, SK (Utility model), TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).

Published
With international search report.

(54) Title: RECOMBINANT STAPHYLOCOCCAL NUCLEASES AND THEIR USE IN LIMITING THE SURVIVAL OF GENETICALLY ENGINEERED MICROORGANISMS



(57) Abstract

The survival of cells can be limited by introducing into the cells a regulatably expressible gene whose expression results in the formation of a cytoplasmatically active truncated and/or mutated *Staphylococcus aureus* nuclease. Populations of cells containing, in addition to such a regulatably expressible gene, a DNA coding for an immunologically active, pesticidally active or environmental pollutant-degrading gene product are useful in immunologically active compositions, pesticidally active compositions and environmental pollutant-degrading compositions, respectively.

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RECOMBINANT STAPHYLOCOCCAL NUCLEASES AND THEIR USE IN LIMITING THE SURVIVAL OF GENETICALLY ENGINEERED MICROORGANISMS

FIELD OF INVENTION

The present invention provides a novel system of biological containment of genetically engineered microorganisms. In particular, the invention relates to novel recombinant staphylococcal nucleases; to a cell or a population hereof containing a regulatably expressible gene whose expression results in the formation of the cytoplasmatically active staphylococcal nuclease at a rate which leads to cell function limitation; to a recombinant replicon containing such a regulatably expressible gene; to methods of limiting the survival of a population of cells containing the expressible gene, to a method of containing an extrachromosomal recombinant replicon to a first kind of cells and a method of stochastically limiting the survival of a cell population.

There is also provided an immunologically active composition, a pesticidal composition and an environmental pollutant-degrading composition, all of which contain a cell population as defined above wherein the cells further contain a DNA sequence coding for a gene product which is immunologically active, pesticidally active or environmental pollutant-degrading, respectively.

TECHNICAL BACKGROUND

The increasing application of recombinant DNA technology to engineer novel microorganism which are industrially useful have caused concerns in both the scientific community and the general public over potential risks. These concerns are primarily related to the potential harm to humans and to undesirable and/or uncontrollable ecological consequences upon deliberate or unintentional release of such genetically engineered microorganisms (GEMs) into the environment. These

concerns have led to the establishment of official guidelines for the safe handling of GEMs in laboratories and production facilities where such organisms are applied. Up till now, such guidelines have primarily been directed to measures of physically containing GEMs in laboratories and production facilities with the aim of reducing the likelihood that workers in such facilities were contaminated, or that the GEMs were to escape from their primary physical environment, such as a fermentation vessel.

It is presently being recognized that the level of safety in the handling of GEMs can be increased by combining physical containment measures with biological containment measures to reduce the possibility of the survival of the genetically engineered organisms if they were to escape from their primary environment.

Lately, however, concerns have become increasingly focused on potential risks related to deliberate release of GEMs to the outer environment and to the use of GEMs as live vaccines. In this connection there is a strongly felt need to have biological containment systems which subsequent to the environmental release of the GEMs or their administration as vaccines to a human or an animal body, effectively kill the released organisms in a controlled way or which limit the function of the released GEMs to an extent where such GEMs are placed at a significant competitive disadvantage whereby they will eventually be ousted by the natural microflora of the environment to which they are released.

The first systems of biological containment were based on the use of "safe" cloning vectors and debilitated host bacteria. As examples, it has been suggested to select vectors which lack transfer functions or which naturally have a very narrow host range. Examples of debilitated host bacteria are *E. coli* mutants having an obligate requirement for exogenous nutrients not present or present in low concentrations outside the primary environment of the GEMs.

Other suggested biological containment systems have been based on mechanisms whereby the vector is restricted to the GEMs e.g. by using a plasmid vector with a non-sense mutation in a gene, the expression of which is indispensable for plasmid replication or a suppressor mutation in the chromosome, said mutation blocking translational read-through of the message of the gene. A further approach is to maintain the rDNA stably in the host by integrating it into the chromosomes of the GEMs.

Recently, an alternative biological containment strategy has been developed in which the recombinant vector is endowed with a gene encoding a cell killing function which gene is under the control of a promoter only being expressed under certain environmental conditions, such as conditions prevailing in an environment outside the primary environment of the GEMs, or when the vector is unintentionally transferred to a secondary host, or the expression of which is stochastically induced. By using incorporation in a GEM of such a cell killing function and selecting appropriate regulatory sequences, vectors can be constructed which are contained in the primary host cell and/or in a primary physical environment. A cell killing function as hereindefined may also be referred to as an active biological containment factor.

If a stochastically induced mechanism of expression regulation is selected for such a biological containment system, a population of GEMs containing the system will, upon release to the outer environment, or if used as a live vaccine be subjected to a random cell killing which will lead to an increase of the doubling time of the host cell population or eventually to the disappearance of the organisms.

The above-mentioned genes encoding cell killing functions are also frequently referred to as "suicide" genes, and biological containment systems based upon the use of such genes, the expression of which are regulated as defined above, are commonly described as conditional lethal systems or "suicide"

systems. Up till now, several cell killing functions have been found in bacterial chromosomes and in prokaryotic plasmids. Examples of chromosomal genes having cell killing functions are the *gef* (Poulsen et al., 1990) and *relF* (Bech et al., 1985, Embo, vol.4 no.4, 1059-1066) genes from *E.coli* K-12. Examples of plasmid encoded suicide genes are *hok* and *flm*A (Gerdes et al., 1986) genes isolated from plasmids R1 and F, respectively, the *snr*B gene also isolated from plasmid F (Akimoto et al., 1986) and the *pnd* gene isolated from plasmids R16 and R483 (Sakikawa et al., 1989 and Ono et al., 1987). Common features of these genes are that they are transcribed constitutively, regulated at a post-transcriptional level, and that they all encode small toxic proteins of about 50 amino acids. The application of the *hok* gene in a biological containment system has been disclosed in WO 87/05932.

Ideally, the features of an effective biological containment system should include as a minimum requirement that the cell killing function when expressed is effective, that the containment system is functional in a broad range of species of GEMs, that the risk of elimination of the cell killing function e.g. by mutations in the suicide gene or the sequences regulating the expression of the gene, is minimal and that the risk of uptake by other organisms of rDNA released when cells are killed is reduced.

None of the above-mentioned known containment systems fulfil all of these ideal requirements. However, in applicants' copending application WO 93/20211 there is provided an active biological containment system which is not based on a primary cell killing function but which makes use of genes, the expression of which in a cell where the gene is inserted, results in the formation of mature forms of exoenzymes such as nucleases derived from gram-negative or gram-positive bacteria or phospholipases which are hydrolytically active in the cytoplasm of the cell and which can not be transported over the cell membrane. When such enzymes are expressed in a

cell, the normal function of the cell becomes limited to an extent whereby the competitiveness, and hence the survival and the environmental persistence, of a population of such cells are reduced significantly.

However, it has been observed that although a bacterial nuclease encoded by a gene from which the sequence coding for the signal peptide has been totally removed may be useful in a method of limiting the survival of GEMs in accordance with the above, a certain transport of the nuclease out of the cell comprising such a gene without the signal sequence-coding sequence may cause "leakage" of active enzyme out of the cell, thereby reducing the efficiency of this containment system. It has now surprisingly been found that this problem can be overcome if a truncated and/or mutated form of a nuclease is used. Additionally, a significant increase in the cytoplasmic enzymatic activity of such a modified enzyme relative to the parent enzyme has been provided thus resulting in improved methods of limiting the survival of GEMs.

Provided the hydrolytically active enzyme is a RNA-degrading and/or DNA-degrading enzyme, a biological containment system based upon such an enzyme may have a further advantage over the known biological containment systems in that the rDNA molecules in a genetically engineered host cell is destroyed simultaneously with the genetically altered host microorganisms. As an example, a recombinant staphylococcal nuclease according the invention having endonucleolytic activity will when used in accordance with the invention cause hydrolysis of the DNA and the RNA to 3'-phosphomononucleotides. However, it may from a GEM risk point of view be advantageous to provide the GEM containment system in a form where further intracellular degradation of the damaged nucleic acids is provided. This may e.g. be provided by inserting into the GEMs a further gene coding for a 3'-5' exonucleolytically active exonuclease. The simultaneous expression and the successive degradative activities of a endonuclease such as the above modified staphylococcal nuclease and an exonuclease

will cause complete destruction of the host nucleic acids, thus killing the host strain without having its nucleic acids remaining stable in the environment for an unlimited period of time.

SUMMARY OF THE INVENTION

Many cells produce hydrolytically active enzymes which are inherently translocated extracellularly, i.e. the enzymes are excreted over the cell membrane. When expressed intracellularly in a cell naturally producing such enzymes, the enzymes are in the form of enzymatically inactive, immature exoenzyme molecules (proenzymes) comprising a signal peptide by means of which the proenzyme is transportable over the cell membrane. On passage of the cell membrane, the signal peptide is cleaved off the molecules which are thereby turned into the mature, enzymatically active form.

The present invention is based on the discovery that a modified, i.e. a truncated and/or mutated form of *Staphylococcus aureus* nuclease being an exoenzyme which normally, while present in the cytoplasm of cells producing them, are in the form of the immature, enzymatically inactive proenzyme may be expressed in a cell in the form of a truncated and/or mutated form of the mature enzyme which is enzymatically active in the cytoplasm of the cell and that the presence intracellularly of such active forms of such modified staphylococcal nucleases may cause damages to the cells leading to a limitation of the normal cell function.

On the basis of these findings, the present invention provides a novel improved approach to biological containment of GEMs aiming at limiting the survival hereof in their environment.

Thus, in a first aspect, the present invention provides a cell containing a gene whose expression results in the formation of a truncated and/or mutated *Staphylococcus aureus* nuclease which is present and hydrolytically active in the cytoplasm of said cell, the cell further containing a regulatory nucleotide sequence which regulates the expression of the gene, the expression of said gene leading to formation of the enzyme in the cell at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cell, to an extent whereby the function of the cell is being limited.

In a further aspect, there is provided a recombinant replicon containing a regulatably expressible gene which, when expressed in a cell encodes a truncated and/or mutated *Staphylococcus aureus* nuclease which is present and hydrolytically active in the cytoplasm of the cell, the expression of said gene leading to formation of the enzyme in the cell at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cell, to an extent whereby the function of the cell is being limited, the expression of said genes being regulated by a regulatory nucleotide sequence which is contained in the recombinant replicon or in an other recombinant replicon present in a cell containing the replicon.

In a still further aspect, the present invention relates to a population of cells consisting of a multiplicity of cells as defined above.

Additionally, the present invention relates to a method of limiting the survival of a cell population in a first or a second environment which method comprises transforming the

cells of said population with a recombinant replicon being replicated in the cells of the population and containing a gene whose expression results in the formation of a truncated and/or mutated *Staphylococcus aureus* nuclease which is present and hydrolytically active in the cytoplasm of said cell, the cells further containing a regulatory nucleotide sequence being regulatable by an environmental factor and which regulates the expression of said gene, the expression of said gene leading to formation of the enzyme in the cells at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cell, to an extent whereby the function of the cells is being limited leading to a limitation of the survival of the cell population.

In another aspect, the invention relates to a method of containing an extrachromosomal recombinant replicon to a first kind of cell, where said replicon is naturally transferable to a second kind of cell, which method comprises providing on the recombinant extrachromosomal replicon a gene whose expression results in the formation of a truncated and/or mutated *Staphylococcus aureus* nuclease which is present and hydrolytically active in the cytoplasm of a cell, the formation of said enzyme being at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cell, to an extent whereby the function of the cell is being limited, said first kind of cells having or being modified to have a chromosomal replicon comprising a regulatory nucleotide sequence which inhibits the expression of said gene and thereby protects said first kind of cells, said regulatory gene being lacking in said second kind of cell, whereby, if a cell of the second kind receives said extrachromosomal recombinant replicon said gene is expressed and has a function-limiting effect thereon.

In another further aspect, the present invention pertains to a method of stochastically limiting the survival of a cell population which comprises transforming the cells thereof

with a recombinant replicon containing a regulatably expressible gene which, when expressed in a cell encodes a truncated and/or mutated *Staphylococcus aureus* nuclease which is present and hydrolytically active in the cytoplasm of the cell, the expression of said gene leading to formation of the enzyme in the cell at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cells, to an extent whereby the function of the cells is being limited, the expression of said genes or genes being stochastically induced as a result of recombinational excision of an excisable negatively functioning regulatory nucleotide sequence which, while present in the cells, inhibits expression of the gene coding for the enzyme, said negatively functioning regulatory nucleotide sequence being contained in the recombinant replicon or in an other recombinant replicon present in cells of the population containing the replicon.

In a still further aspect, there is provided an immunologically active composition which contains a viable function-limited cell population as defined above wherein the cells contain a further DNA sequence not naturally related to the gene coding for the hydrolytically active enzyme or to the regulatory nucleotide sequence, which further sequence is a sequence coding for an immunologically active gene product, the cells being function-limited to an extent which, when the composition is administered to a human or an animal, allows the cells to express the immunologically active gene product for a period of time and in an amount sufficient to obtain an effective immune response in said human or animal, but which does not allow the cells to persist in the human or the animal.

The present invention also provides a pesticidally active composition which contains a viable cell population as defined herein wherein the cells contain a further DNA sequence not naturally related to the gene coding for the hydrolytically active enzyme or to the regulatory nucleotide

sequence, which further sequence is a sequence coding for a pesticidally active gene product, the cells being function-limited to an extent which, when the composition is administered to an environment containing a pest, allows the cells to express the pesticidally active gene product for a period of time and in an amount sufficient to obtain an effective pesticidal effect in said environment but which does not allow the cells to persist in the environment.

Finally, there is provided an environmental pollutant-degrading composition which contains a viable cell population as defined herein, wherein the cells contain a further DNA sequence not naturally related to the gene coding for the hydrolytically active enzyme or to the regulatory nucleotide sequence, which further sequence is a sequence coding for an environmental pollutant-degrading gene product, the cells being function-limited to an extent which, when the composition is administered to an environment containing a pollutant to be degraded, allows the cells to express said pollutant degrading gene product for a period of time and in an amount sufficient to obtain an effective pollutant-degrading effect in said environment but which does not allow the cells to persist in the environment.

DETAILED DISCLOSURE OF THE INVENTION

The concept of an active biological containment system based on the use of an intracellularly hydrolytically active exoenzyme which has a cell function-limiting effect

A number of macromolecules such as proteins, lipids, phospholipids, DNA, RNA and polysaccharides that cannot pass cell membranes are nevertheless utilizable as substrates for cell growth. These substrates are enzymatically hydrolysed (degraded) in the external medium of the cells by protein enzymes excreted by the cells. Such hydrolytic enzymes that mediate extracellular degradation are termed "exoenzymes". In addi-

tion to true exoenzymes which are capable of passing the outer cell membrane and hence excreted into the external medium, certain cells produce incompletely excreted hydrolytic enzymes of which some enter the cell membrane and remain there rather than passing through. Others pass through the cell membrane but not the outer membrane (periplasmic enzymes) and still others remain in the outer membrane.

In the present context, the term "exoenzyme" is used to designate a hydrolytic enzyme which have been excreted naturally by the cell natively producing the enzyme or a cell in which the genes coding for such an excretable enzyme has been inserted, by passing through the cell membrane (the cytoplasmic membrane) and accordingly, this term include native true exoenzymes, cell membrane-bound enzymes, periplasmically located enzymes and outer membrane-bound enzymes.

Generally, the mechanisms by which excretable exoenzymes as defined herein are excreted share common features. Thus, when first synthesized in the cytoplasm, these proteins are in the form of enzymatically inactive precursor molecules (also referred to as proenzymes) which are larger than they are after they have entered their proper extracellular location, since a portion of the amino-terminal end of the protein which is termed as the signal peptide, is removed after the protein is properly located. The function of the signal peptide is to aid in the translocation of the protein exoenzyme to its place of location. When the molecule is properly located, the signal peptide is removed whereby the protein is turned into the mature or "processed", enzymatically active hydrolytic enzyme.

As explained above, the present invention presents a novel approach to the concept of active biological containment which is based on the finding that it is possible to obtain in the cytoplasm of a cell, i.e. intracellularly, the presence of enzymatically active mature forms of exoenzymes. This is obtained by the insertion in the cell of a nucleotide

sequence which codes for the exoenzyme molecule, but which does not comprise the sequence coding for the corresponding native signal peptide herefor. By deletion from the nucleotide sequence of the sequence coding for the signal peptide it is also obtained that the intracellularly active enzyme molecule cannot be translocated outside the cell membrane.

The cytoplasm of cells contains a mixture of macromolecules, the presence and function of which are necessary for the non-limited function of the cells, including basic life manifestations such as growth and replication. Examples of such macromolecules include DNA, RNA, lipids, phospholipids, proteins and polymeric carbohydrates.

When, in accordance with the present invention, a gene whose expression results in the formation of an exoenzyme as defined above is inserted into the cell, naturally occurring intracellular macromolecules may act as substrate for the intracellularly active hydrolytic enzyme.

Provided the hydrolytically active enzyme is expressed intracellularly at a rate which results in the hydrolysis of hydrolysable cytoplasmic macromolecules to an extent whereby the life functions of the cells become limited, the competitiveness of such cells with cells of the same type, but in which an exoenzyme as defined herein is not expressed in intracellularly active forms, will be decreased.

In the present context, the term "non-limited cell function" denotes that the growth of a cell as manifested i.a. by the synthesis of new cell material and the rate of replication of the cell is not decreased by an intracellular hydrolytic macromolecule degradation, not natively occurring in the cells. Accordingly, this term is used herein to describe i.a. the growth rate and the rate of replication, under any given condition, of a cell in which an intracellularly active exoenzyme as defined above is not being expressed. Consequently, the term "limited cell function" as used herein

describes, in a relative manner, the state of a cell in terms of i.a. a reduced growth rate and/or reduced rate of replication which results from the expression therein of an intracellularly active exoenzyme when the cell is grown under the same conditions as a cell of the same kind having a non-limited cell function as defined above.

The recognizable manifestation of such limited cell function may ultimately be cell death, but it may also, relative to a cell having a non-limited cell function, be a reduced cell growth appearing as a reduced rate of replication resulting in a reduced increase of cell numbers within a certain period of time as a result of an increase of the lag phase and/or of the cell doubling time. Other manifestations may be a relatively increased requirement for one or more nutrient components or a relatively higher susceptibility to detrimental environmental factors such as sub-optimal temperatures or cell damaging caused by toxic substances.

The actual type of manifestation or manifestations of such limited cell function and the degree hereof will depend in particular on the specific species of the exoenzyme being expressed in the cell, the rate of expression of the exoenzyme, the capability of the enzyme to be hydrolytically active under the conditions prevailing in the cytoplasm, the amounts of substrate macromolecules, and the significance of such macromolecules for non-limited function of the cell, and the number of copies of the gene encoding the intracellularly active exoenzyme.

<u>The intracellularly hydrolytically active exoenzyme having cell function-limiting effect</u>

In accordance with the invention, a suitable intracellularly active exoenzyme may be any truncated and/or mutated *Staphylococcus aureus* nuclease which, when it is expressed, is capable of hydrolytically degrading macromolecules present in

the cytoplasm of prokaryotic and eucaryotic cells to an extent whereby the function of the cell is being limited.

As used herein, the term "nuclease" denotes hydrolytic exoenzymes capable of degrading nucleic acids. The nucleic acids DNA and RNA are polynucleotides formed by the joining of nucleotides by phosphodiester bonds. Some nucleases are capable of degrading both DNA and RNA, whereas others (deoxyribonucleases or DNases) degrade only DNA and still others degrade only RNA (ribonucleases or RNases). Nucleases may either be exonucleases which are diesterphosphate bond-hydrolysing enzymes cleaving off the last nucleotide residue in either of the two terminals of an oligonucleotide, or endonucleases which cleave phosphodiester bonds located in the interior of polynucleotides. In the art, the term "nick" is normally used to describe the lesion in a DNA strand resulting from an endonuclease cleavage of a diesterphosphate bond. It must be noted that term "exonuclease" when used in the present specific context denotes the mode of action as defined above and thus, the term is not used here to designate a nuclease normally being excreted out of the cell.

In the context of the present invention, useful nucleases include staphylococcal nucleases having the capability of hydrolysing diesterphosphate bonds in DNA. Useful DNA-degrading nucleases include endonucleases, the activity of which, when double-stranded DNA is the substrate, results in the cleavage of diesterphosphate bonds (nicks) in only one of the strands of the DNA. Accordingly, in one embodiment of the invention, the hydrolytically active enzyme is a staphylococcal endonuclease having such capability.

Lesions in the DNA occur spontaneously in all cells with a relatively high frequency. However, in the normal cell such lesions are efficiently eliminated by an inherent DNA repair mechanism which involves that the altered portion of the damaged strand is recognized and removed by one set of enzymes and then replaced in its original form by a DNA poly-

merase, and finally an enzyme called DNA ligase seals the nick (the broken diesterphosphate bond) that remains in the DNA helix, to complete the restoration of an intact DNA strand.

This natural cell DNA repair mechanism will also eliminate DNA lesions (nicks) which may result from the enzymatic activity in a cell of a nuclease as defined herein. Accordingly, the function of a cell in which the intracellularly active nuclease as defined above, is expressed will only be limited when the nuclease is expressed at a rate which results in the presence of nicks in the cell nucleic acids in a number exceeding the number which can be repaired by the DNA repair mechanism, so that the number of DNA lesions (nicks) reaches a level whereby the function of the cell becomes recognizably limited.

The rate of expression of the intracellularly active exoenzyme as defined herein is also determined by the specific activity of the enzyme under the conditions prevailing in the cytoplasm of the cell. It will be understood that these conditions may differ from those prevailing in the extracellular environment into which the enzyme when present in a cell where it is normally produced, is translocated. As one example, an enzyme comprising sulphur-containing amino acid residues such as cysteine and methionine will be prone to damages caused by oxidation and hence, it may be assumed that it will be at least partially enzymatically inactive when present in the cytoplasm of a cell where the E_h-value is high. However, it was surprisingly found that an enzyme comprising sulphur-containing amino acids may be enzymatically active intracellularly to an extent whereby the function of a cell in which the enzyme is expressed, is limited.

Several prokaryotic cells including gram-negative and gram-positive bacteria inherently produce exoenzymes which are translocated over the cell membrane, interesting examples being endonucleases produced by *Serratia* spp. It is known

that such endonucleases have a high specific activity extracellularly. In WO 86/06743 are disclosed such extracellular *Serratia* spp. nucleases being expressed in *E. coli* and their usefulness in the removal of nucleic acids from biological materials is described. In WO 93/20211 is disclosed a *Serratia* spp. nuclease which when expressed intracellularly in the form of the mature enzyme, i.e. without the signal peptide, is enzymatically active to an extent whereby the function of the cell in which the enzyme is expressed, may be limited.

The present invention pertains to a hydrolytically active enzyme which is a truncated and/or mutated form of a nuclease encoded by a gene isolated from a gram-positive bacterial species such as a *Staphylococcus* species, one suitable example hereof being the mature form of the nuclease of the Foggi strain of *Staphylococcus aureus* (ATTC 27355). The gene encoding this enzyme has been cloned and characterized (Shortle, 1983, Gene, 22, 181-189). This nuclease is a Ca^{2+}-activated 16.8 kD thermostable extracellular phosphodiesterase which degrades both DNA and RNA. The enzyme consists of 149 amino acids with no disulphide bonds or free sulfhydryl groups. The gene encoding this wildtype nuclease may be derived from plasmid pFOG408 or plasmid pFOG301 containing the gene.

During the experimentation leading to the present invention, it has been found that a staphylococcal nuclease including the above nuclease of the *Staphylococcus aureus* (Foggi) strain may be hydrolytically active even in a truncated form, i.e. in a form where amino acid residues have been deleted from the N-terminal or the C-terminal end of the wild type enzyme, or from both ends. Examples of such truncated hydrolytically active enzymes are nucleases encoded by the plasmids pSNUC24-26 and pSNUC420-26 as described hereinbelow. Such truncated enzymes may have improved characteristics as compared to the parent enzymes from which they are derived. Such improved characteristics may include higher specific

activity, higher intracellular stability, no or very low transport of the enzyme out of the cell.

Accordingly, in other useful embodiments of the present invention the hydrolytically active enzyme is in a truncated form of a staphylococcal endonuclease, e.g. lacking at least 9 amino acid residues of the parent enzyme from which it is derived.

Such a truncated staphylococcal nuclease may even maintain its enzymatical functionality when further modified relative to the parent enzyme. Thus, such a truncated nuclease may be mutated in one or more codons by substitution of at least one nucleotide or it may be modified by insertion of DNA sequences in the coding sequence for the truncated enzyme. Furthermore, it was found that deletions of one or more nucleotides may occur in the coding sequence without impairing the function of the enzyme, even when such a deletion causes a frameshift mutation.

A cell containing the hydrolytically active exoenzyme

As mentioned above, the present invention relates in one aspect to a cell containing a gene coding for the hydrolytically active exoenzyme as defined herein. The cell may be selected from a wide variety of cells for which a need for containment exists. Thus, the cell to be contained may be a bacterial cell, a protozoan cell, a yeast or fungal cell, or a cell derived from the tissues of multicellular organisms such as plants, animal and fungi.

The gene coding for the hydrolytically active enzyme may be derived from a variety of replicons contained in any *Staphylococcus* organism producing an extracellular enzyme as presently defined. Thus, sources of the gene include staphylococcal chromosomes or plasmids. The gene may also be constructed synthetically according to standard procedures.

In accordance with the invention, the cell as defined herein may be obtained by methods known *per se*. These methods include the steps of screening for cells expressing a suitable extracellular hydrolytic enzyme, isolating from such cells the gene encoding the enzyme, removing from the gene the nucleotide sequence coding for the signal peptide and, if desired, removing part of the coding sequence for the mature form of the nuclease, and/or mutating the gene in one or more codons, and inserting into a cell to be contained, the thus modified gene and a nucleotide sequence capable of regulating the expression hereof, growing the cell under conditions allowing the expression of the gene, and testing for the intracellular presence of the enzyme in an enzymatically active form.

The insertion of the gene coding for the hydrolytically active exoenzyme may be in the chromosome of the cell or the gene may be inserted in an extrachromosomal recombinant replicon such as a recombinant plasmid capable of replicating in the cell.

<u>The regulatory nucleotide sequence regulating the expression of the hydrolytically active exoenzyme</u>

As mentioned above, the expression of the gene coding for the hydrolytically active enzyme is regulated by a regulatory nucleotide sequence. In the present context the term "regulatory nucleotide sequence" is intended to indicate a nucleotide sequence which directly or indirectly regulates the expression of the gene coding for the hydrolytically active enzyme at the level of transcription or at the level of translation. The regulatory nucleotide sequence may be one, the function of which results in a suppression or inhibition of the activity of the regulatable promoter. Such regulatory nucleotide sequences are herein referred to as "negatively functioning regulatory nucleotide sequences".

One interesting example of such a negatively functioning regulatory nucleotide sequence is a sequence coding for a repressor substance which represses the expression of the gene coding for the hydrolytically active enzyme and which substance may, when a cell containing it is released to a human or an animal body or to the outer environment, undergo a decay whereby the repression of expression of the enzyme-encoding gene is gradually reduced and eventually, when the decay of the repressor is completed, the repression is removed.

In preferred embodiments of the invention, the regulatory nucleotide sequence may be contained in the cell in one or more recombinant replicons and it may be contained in the same replicon as that containing the enzyme-encoding gene or in a different recombinant replicon.

One way whereby the expression of the cell function-limiting enzyme may in accordance with the invention be regulated is by providing in the cell a gene coding for the hydrolytically active enzyme, which gene is regulated at the level of transcription. The regulation at the level of transcription may be carried out in various ways including a regulation by means of a promoter, regulated by one or more factors. These factors may either be ones which by their presence ensure expression of the gene coding for the cell function-limiting enzyme or may, alternatively, be ones which suppress the expression of the gene so that their absence causes the enzyme to be expressed.

Factors regulating the activity of the promoter as defined above may be selected from a variety of factors. Thus, the expression of the gene encoding the cell function-limiting enzyme may be determined by the environmental conditions or the physiological state of the cells, or by a cyclical or stochastic event. In the present context, the term "cyclical event" is understood to mean a cyclically recurrent event causing changes in certain factors known to be potentially

useful in influencing the expression of genes such as temperature conditions, changes in light intensity or hormonal changes. The term "physiological state of the cells" denotes factors such as cell density or the growth phase of cells.

In accordance with the invention, advantageous promoter regulating factors are readily regulatable factors including the presence or absence of a certain chemical substance in the environment or the physical conditions in the environment such as the prevailing temperature or other physical factors (e.g. the intensity of the light in the environment). Thus, it is possible to envisage containment systems as presently claimed, in which the gene coding for the cell function-limiting enzyme is expressed when a certain chemical substance present in a first environment such as the fermentation medium in which the cell is propagated, is not present in a second environment to which the cell is released, or when a factor required for the growth or survival of the cell is no longer present, or the factor is a factor which, when it is exhausted from an environment of the cell, has the desired effect, viz. that the gene is expressed.

The promoter regulating the transcription of the gene coding for the cell function-limiting hydrolytically active enzyme may also become activated in a second environment of the cell by a chemical substance which is not present in a first environment of the cell, but which is present in the second environment in sufficient quantities to activate the promoter. Similarly, the promoter may by a promoter which is activated by a shift in temperature, such as a shift from a higher temperature in a first environment as e.g. a fermentation vessel, to a lower temperature prevailing in an outside second environment, or the intensity of light, in that the promoter may be one which is activated in the presence of light of sufficient intensity, but is inactive in the darkness prevailing in a first environment such as a fermentation vessel.

Where cells as defined herein are ones that are to be released to the natural environment in a controlled manner, e.g. to a restricted area of land or to the intestinal tract of a human or an animal, the regulatable promoter may be one which is regulated chemically, i.e. by the presence or absence of a certain chemical substance in the environment of the cells as it has been explained above.

However, the regulatable promoter is advantageously a promoter which is activated cyclically, e.g. by changes of the temperature, or most advantageously by a stochastic event. The term "stochastic event" as used herein is intended to denote an event which occurs at random at a certain frequency per cell per generation or frequency per unit time which, in accordance with the invention may result in a limitation of the function of the cells in which the activation of expression of the cell function-limiting intracellularly active exoenzyme occurs, optionally to an extent which leads to the death of the cells. The stochastic event may be occasioned by periodic inversions of the region carrying the promoter, but is more advantageously induced by the recombinational excision of a recombinationally excisable negatively functioning regulatory nucleotide sequence as defined above.

It should be noted that in order to ensure a general applicability of the present invention, the promoter used to initiate transcription of the gene coding for the cell function-limiting enzyme is preferably a promoter which is capable of causing expression of said gene in a wide range of cells.

In case of regulatable transcription of the hydrolytically active enzyme, the regulatory nucleotide sequence may e.g. be a promoter isolated from bacterial operons involved in the biosynthesis of amino acids or from bacterial genes, the transcription of which is activated late in the stationary growth phase or from bacterial genes involved in the synthesis of cell surface structures such as fimbriae. Examples of suitable promoters are *E. coli trp* which becomes activated

in the absence of tryptophan, the bacteriophage λ P_R and P_L promoters controlled by temperature sensitive regulatory nucleotide sequences, the *Bacillus subtilis* sporulation gene promoters which are activated during sporulation, and the *E. coli* and *Salmonella* fimbriae gene promoters which are activated stochastically.

In case of chemically regulatable promoters, the chemical substance, the presence or absence of which determines the activation of the promoter, may suitably be selected from carbon or nitrogen sources, metabolites, amino acids, nucleosides, purine or pyrimidine bases or metal ions. When the chemical substance is one which, when present, suppresses promoter activity, it should preferably be a substance which rarely occurs in the natural environment in such concentrations that the promoter would not be activated when the cell is released to the natural environment. One example of a suitable promoter in e.g. an *E. coli* cell is the *trp* promoter which is repressed in the presence of tryptophan in the environment of the cell, but which is derepressed in the absence of sufficient amounts of tryptophan in the environment. A containment system using the *trp* promoter or another promoter being regulated in the same manner, might therefore comprise an amount of tryptophan in a first environment, such as a fermentation vessel, to repress the promoter which is derepressed when the cell is released from the first environment to a second environment, e.g. the natural environment which usually contains very low amounts of tryptophan or no tryptophan at all.

Another example of a regulatable promoter, the activation of which is determined by a chemical substance is the *lac* promoter which is inducible by e.g. isopropyl-β-D-thiogalactopyranoside (IPTG).

Where cells as defined herein are used in a pesticidally active composition e.g. being administered to a plant, the

regulatable promoter may suitably be regulated by the presence/absence of a compound secreted by this plant.

As mentioned above, the regulatable promoter may be a promoter, the activity of which is determined by the temperature prevailing in the environment of a cell containing the gene coding for the cell function-limiting enzyme and a the regulatable promoter regulating the expression of the gene. In such a case, the regulation of the promoter is advantageously obtained by the presence in the cell of a temperature sensitive gene coding for a repressor for the promoter. As one typical example, the λ promoters including those mentioned above may be regulated by a temperature sensitive λ cI repressor.

Promoters which are activated stochastically by periodic inversions of the promoter region (in the present context, such promoters are also termed as an "invertible promoter" and "inversional switch promoter") and which may be useful for the purposes of the present invention include as examples the *hin*, *cin* and *gin* promoters. One particularly useful invertible promoter is the *fimA* promoter which is one *E. coli* fimbriae promoter. The activation (inversional switch) of this promoter is regulated by the gene products of the two genes which for the present purposes is termed the "on" and the "off" genes, the *on* gene product inducing a switch from off (inactive) to on (active), and the *off* gene product inducing a switch from on to off. In a wild-type *E.coli* cell where the *fimA* gene and its associated promoter is present in one copy on the chromosome, the inversional switch occurs with a switching frequency of about one cell/1000 cells/ generation. It is, however, possible to regulate the frequency of the inversional switch as required by regulating the dosage of expression of the *on* and *off* genes. This may e.g. be effected by means of suitable promoters to transcribe into the *on* and *off* genes. The frequency of transcription initiation by these promoters will then determine the rela-

tive dosage levels of the *on* and *off* gene products being formed.

As it has been explained above, the intracellular enzymatic activity of the cell function-limiting enzyme may also be regulated by the presence or absence in the cell of a gene product rendering the cell refractory to said enzymatic activity, e.g. by interacting with the enzyme so that the cell function-limiting effect hereof is not expressed.

Stochastically induced regulation of the gene coding for the hydrolytically active exoenzyme by means of recombinational excision of negatively regulatory nucleotide sequences

In accordance with the invention, one particularly advantageous method of stochastically regulating the expression of the gene coding for the cell function-limiting exoenzyme is the induction of the gene expression as a result of recombinational excision of an excisable negatively functioning regulatory nucleotide sequence which, while present in the cell, inhibits expression of the gene. In the present context, the term "recombinational excision" refers to the result of a naturally occurring phenomenon of genetic recombination (cross-over) whereby nucleotide sequences in replicons, in a controlled process, pair, brake and rejoin to form recombinant replicons by the sequential action of enzymes acting on the DNA. The frequency of recombinational events in a cell depends i.a. on the degree of homology between paired complementary nucleotide sequences and on the length of the complementary sequences. Thus, it has been shown that about 50 base pairs of homology may be required to obtain recombination in a bacterial cell.

When a negatively regulatory nucleotide sequence is inserted between directly repeated nucleotide sequences of a sufficient length in a recombinationally proficient cell which, in accordance with the invention contains a gene coding for a cell function-limiting enzyme, recombination between the

repeats results in the recombinational excision of the negatively regulatory nucleotide sequence allowing the gene to be expressed, whereby the cell function-limiting enzyme may be produced intracellularly in amounts leading to a limitation of the cell function, optionally resulting in the death of the cell.

Accordingly, the phenomenon of recombinational excision as used herein, implies that a DNA subsequence, i.e. the negatively regulatory nucleotide sequence, is excised from a longer DNA sequence through a recombination event. In essence, the longer DNA sequence is cleaved on either side of the subsequence and the fresh ends are joined, leaving out the subsequence. Recombination occurs between sufficient homologous flanking nucleotide subsequences. Thus, with DNA of the general structure W-X-Y-X-Z, X being a repeated sequence and Y being a negatively regulatory nucleotide sequence, this could recombine to form W-X-Z, with the Y subsequence being excised.

As mentioned above, the frequency of the recombination can to some extent be determined by varying the lengths of the repeats and/or the distance between the repeats. Furthermore, the frequency may be varied by using repeat sequences of varying homologies. Thus, nucleotide sequence repeats being 100% homologous and having a size which does not impair recombination will result in a high recombination frequency and hence, in a high frequency of recombinational excision of the negatively regulatory sequence, whereas mismatches within complementary sequences will reduce the recombination frequency depending on the degree of mismatch. As an example, it has been found that 10% divergence between nucleotide sequence repeats may reduce the recombination frequency 40-fold.

Accordingly, the cell containing the gene coding for a hydrolytically active exoenzyme may, in accordance with the invention, be a cell containing a regulatory nucleotide se-

quence which is a recombinationally excisable negatively functioning regulatory nucleotide sequence being flanked by a first flanking nucleotide sequence and a second flanking nucleotide sequence substantially homologous with the first flanking sequence. As used herein, the term "substantially homologous with" is used to indicate that the degree of homology is sufficient to result in a desired frequency of recombination. In certain embodiments it may, in order to obtain a desirable maximum frequency of recombination, be advantageous to use direct repeats, i.e. sequences being 100% homologous, whereas it may, in other embodiments where a moderate degree of cell function limitation is desirable, be appropriate to use repeats which are more or less heterologous, but still allowing a desirable lower frequency of recombination to occur. Accordingly, in the present context, the term "sufficiently homologous" may appropriately be used to indicate a degree of homology between two flanking nucleotide sequence repeats which results in a desired frequency of recombinational events in a cell containing the gene coding for the hydrolytically active exoenzyme and a negatively regulatory nucleotide sequence.

As it also has been mentioned above, the frequency of recombination depends on the lengths of the flanking sequences. In useful embodiments of the invention, flanking sequences are used which have a length being in the range of 100-5000 base pairs. In certain preferred embodiments, it may be advantageous to use flanking sequences, the length of which is in the range of 200-3000 base pairs. As the flanking sequences may be used any nucleotide repeats of sufficient lengths and homology as it has been defined above. As one useful example of flanking sequences may be mentioned the chloramphenicol resistance gene having a size of about 900 base pairs and which occurs in the plasmid pBR325 (Bolivar, 1978, Gene, 4, 121-136). Another example of a useful nucleotide sequence which when inserted as repeats may result in recombination, is a subsequence of the *rrnB* gene isolated from the plasmid pKK3535 (Brosius et al., 1981, Plasmid, 6, 112-118) of the

rrnB gene isolated from the plasmid pKK3535 (Brosius et al., 1981, Plasmid, 6, 112-118) having a size e.g. in the range of 500 to about 3000 base pairs, such as 598 base pairs.

In one interesting embodiment of the invention, the cell containing a gene whose expression in the cell results in the formation of a hydrolytically active enzyme is a cell wherein said gene is a gene which encodes a first RNA which is a messenger RNA, and which further contains an excisable negatively regulatory nucleotide sequence operably linked to said gene encoding the hydrolytically active gene, which is a gene encoding a second RNA which forms an RNA-RNA duplex with said first messenger RNA and thereby, when it is expressed, inhibits translation of said gene coding for the hydrolytically active enzyme.

In another useful embodiment of the present invention, the recombinationally excisable negatively regulatory nucleotide sequence is a gene encoding a polypeptide repressor of transcription of the gene whose expression results in the intracellular formation of a hydrolytically active enzyme. Such a polypeptide repressor may, e.g. be a *lac* repressor. As one specific example of a useful *lac* repressor may be mentioned the repressor encoded by the *LacIq* gene.

In a further useful embodiment of the invention, the excisable negatively regulatory nucleotide sequence is a transcription termination sequence, preventing the transcription of the gene whose expression results in the formation intracellularly of a hydrolytically active enzyme. In one specific embodiment of the invention, such a suitable terminator sequence may be the *rpoCt'* transcription terminator isolated from the plasmid pHBA102rpoCt (Squires et al., 1981, Nucleic Acid Res., 9, 6827-6839).

Negatively regulatory nucleotide sequences which in accordance with the invention are suitable, may be isolated from nucleotide sequences derived from a virus, or a prokaryotic

or eucaryotic cell. Thus, sources of the nucleotide sequence include bacterial chromosomes, bacterial plasmids, prokaryotic viruses, eucaryotic viruses, eucaryotic plasmids, or eucaryotic chromosomes.

In preferred embodiments of the invention, the excisable negatively regulatory nucleotide sequence being operably linked to the gene coding for the hydrolytically active enzyme and the first and second flanking sequences, both as defined above, is provided in the form of a "cassette" which term is used herein to describe a readily insertable nucleotide sequence comprising at least the above-mentioned sequences and optionally the gene coding for the hydrolytically active enzyme, and optionally further nucleotide sequences including as examples a suitable marker such as a gene coding for antibiotic resistance. In the present context, the term "insertable" denotes that the cassette as defined herein is provided with suitable restriction sites at both ends allowing for insertion in a replicon having the same restriction sites. Accordingly, such preferred restriction sites include sites which occur frequently in replicons where insertion is desirable or alternatively, restriction sites which may be easily provided in such replicons.

It will be understood that, in accordance with the invention, a cassette as defined above and which does not comprise the gene coding for hydrolytically active enzyme and operably linked to the negatively regulatory nucleotide sequence, may be inserted in a replicon which is different from the replicon containing said gene. Optionally, the cassette as defined above may be inserted in a first replicon such as e.g. a transposon and subsequently inserted via the transposon into the chromosome to obtain a cell as defined herein.

As it has been explained above, the activation of certain invertible promoters such as the *fimA* promoter or functional homologues hereof is regulat d by the gene products of an *on* gene and an *off* gene. It will be understood that this mechan-

ism of promoter regulation provides the possibility of using the *off* gene or a functional homologue hereof as a negatively regulatory nucleotide sequence which may be inserted in the cell as defined herein, as a recombinationally excisable nucleotide sequence in the manner explained in details above. Accordingly, in one embodiment, the present invention provides a cell wherein the gene whose expression results in the formation of a hydrolytically active enzyme is stochastically expressed as a result of recombinational inversion of an invertible promoter sequence of the regulatory nucleotide sequence said promoter being operably linked to the gene.

Stochastically induced regulation of the gene coding for the hydrolytically active exoenzyme by means of site-specific recombinational excision of negatively regulatory nucleotide sequences

In plasmids, inherent mechanisms occur whereby multimer resolution of the plasmid during replication takes place. As exemplified by the broad host range plasmid RP4, this resolution system may comprise (1) a gene coding for a multimer resolving enzyme, a resolvase and (2) a site for the site-specific resolvase-mediated resolution. In plasmid RP4 the gene coding for the resolvase is *parA* and the site for the resolution is designated mrs. If two mrs sites are placed in direct orientation, a nucleotide sequence inserted between those two sites may, if the *parA* gene is present in the same host cell, be deleted at a relatively high frequency whereby a site-specific recombination system is provided. In useful embodiments the *parA* gene may be located in *trans*.

It has now been found that such a site-specific recombination system provides a useful mechanism for stochastically regulating the expression of a gene such as the gene coding for the hydrolytically active enzyme as defined herein, since the site-specific recombination may be used to obtain recombinational excision of a negatively regulatory nucleotide sequence as defined above.

Accordingly, in one interesting embodiment, the present invention provides a cell as defined herein in which the negatively regulatory nucleotide sequence is a sequence flanked by a first site for a site-specific resolution recombinase and a second site for site-specific resolution, the second site being recognizable by the same or a functionally equivalent multimer resolving enzyme as is the first site, whereby the regulatory sequence is recombinationally excisable in the cell. In a specific embodiment, the gene coding for the multimer resolving enzyme is located in *trans* relative to the sites for site-specific resolution. In the present context, one useful example of a suitable gene is the *parA* gene isolated from plasmid RP4.

Provision of a cell according to the invention in which the function-limiting effect of the hydrolytically active enzyme is increased by mutation

In certain embodiments, it may be advantageous to obtain a higher intracellular enzymatic activity than is obtained by the insertion of the gene coding for the hydrolytically active enzyme and/or the regulatory nucleotide sequence in the form in which this/these sequence(s) are primarily isolated. Such a higher activity may be the result of an increased amount of enzyme being expressed and/or of an increased specific activity of the enzyme, relative to the amount or the specific activity which is obtained by the insertion of the primarily isolated sequences. An increased intracellular activity of the hydrolytically active enzyme may also be the result of an increased intracellular stability of the enzyme. Furthermore, a higher intracellular enzymatic activity may be obtained with a modified enzyme which relative to a parent enzyme which to some extent is transported extracellularly, is retained essentially intracellularly.

Such increased enzymatic activity may conveniently be obtained by subjecting the isolated sequences separately or

together to a conventional *in vitro* or *in vivo* mutagenization treatment e.g. with a chemical mutagen, by means of a site-directed mutagenesis, e.g. by use of a PCR technique, or by a mutagenically active radiation treatment.

Cells containing thus treated nucleotide sequences in which a mutation or mutations resulting in an increased intracellular enzymatic activity have occurred, may be selected by growing a culture of the cells under conditions where the gene coding for the hydrolytically active enzyme is expressible, and isolating cells or cell clones which relative to cells or cell clones containing the primarily isolated sequences and grown under the same conditions, show an increased level of cell function limitation as defined above.

Accordingly, the invention provides in one embodiment a cell as defined herein in which at least one of the gene whose expression results in the formation of a hydrolytically active enzyme and the nucleotide sequence regulating said gene, is mutated at one or more sites, whereby the cell function-limiting effect of the enzyme encoded by the gene, when expressed in the cell, is the same or increased relative to the cell function-limiting effect of the enzyme expressed in a cell containing said gene and said nucleotide sequence in non-mutated form. In one interesting embodiment, the cell in which the enzyme activity is increased by the mutation treatment is a cell containing a gene coding for a mutant nuclease encoded by a mutated *Staphylococcus* spp gene such as the nucleases encoded by pSNUC24-26 or pSNUC420-26 as described below.

<u>A cell according to the invention comprising a further regulatably expressible gene which encodes a cell function-limiting gene product</u>

As a means of increasing the cell function-limiting effect in a cell as defined herein it may be advantageous to insert into the cell a further regulatably expressible gene which

further gene is a gene encoding a non-enzyme cell function-limiting gene product or a further enzymatically active cell function-limiting gene product. Accordingly, the present invention provides in one specific embodiment a cell containing a further gene as defined above. Such a further regulatably expressible gene may be regulated by a regulatory nucleotide sequence of the same type as the sequence regulating the gene coding for the hydrolytically active enzyme or the gene may be one which is regulated by a regulatory nucleotide sequence of another type, said other type of regulatory sequence optionally being one which is also capable of regulating the expression of the gene coding for cell function-limiting hydrolytically active enzyme.

A suitable gene coding for a further non-enzyme regulatably expressible cell function-limiting gene product may conveniently be selected from the above-mentioned genes having cell killing function. In certain preferred embodiments, said further gene is selected from the *hok* gene of the *parB* region of plasmid R1, the *gef* gene and a DNA sequence which is a functional equivalent of either of these genes.

A suitable further enzymatically active cell function-limiting gene product is an exonucleolytically enzyme which is expressed intracellularly in the mature form including as an example the *E. coli* exonuclease III. One advantage of providing in a cell to be contained the simultaneous expression of the staphylococcal nuclease according to the invention and an exonuclease as defined above is that the enzymatic efficiency of the staphylococcal nuclease in the cell is enhanced.

A cell as defined herein which further contains a DNA sequence not naturally related to the replicon carrying the gene coding for the hydrolytically active enzyme

As it has been defined above, the cell according to the present invention may, when occurring as a population comprising a multiplicity of the cell which further comprises a

DNA sequence coding for an immunologically active enzyme, a pesticidally active or a pollutant-degrading gene product, respectively, be useful in an immunologically active composition, a pesticidally active or a environmental pollutant-degrading composition, respectively, to be contained in the particular environment where such a composition may be released.

Accordingly, the cell may in such specific embodiments be a cell which further comprises a DNA sequence not naturally related to the replicon carrying the gene whose expression results in the formation of a hydrolytically active enzyme and/or the replicon carrying the regulatory nucleotide sequence, said DNA sequence being selected from a sequence coding for an immunologically active gene product, a sequence coding for a pesticidally active gene product and a sequence coding for a pollutant degrading gene product.

In the present context, the term "immunologically active gene product" is used to describe an epitope (antigenic determinant) from a pathogenic organism which, when it is administered to the body of a human or an animal is capable of stimulating the formation of antibodies therein. A cell as defined in the present invention which contains one or more genes encoding such a gene product may be utilized in the preparation of useful live vaccines. In the immunization against several pathogens it is considered advantageous to administer live vaccines as compared to killed organisms or antigenic fragments of the pathogen, since the level of immunity conferred by a live vaccine is frequently higher than that conferred by vaccines comprising killed pathogenic organisms or fragments thereof. Most known vaccines comprising viable epitope-containing organisms are either based on recombinant non-pathogenic organisms encoding the epitope or they are based on attenuated pathogenic organisms. The cell may advantageously contain a multiplicity of genes each of which coding for a specific immunologically active gene product.

However, up till now the use of live vaccines has been limited since it is often difficult to obtain the right combination of attenuation, viability and adequate immune response. Furthermore, the deliberate release of genetically engineered microorganisms to the body and to the external environment which is a result of the use of viable recombinant organisms as vaccines, is currently not allowed in any country for reasons of public concern as to the possible long-term environmental impact, in particular the risk of permanent establishment of the GEMs in the environment.

The present invention provides an advantageous means of circumventing these problems associated with the use of known GEM-based live vaccines by introducing into a viable epitope-containing cell the regulatably expressible gene coding a cell function-limiting hydrolytically active enzyme as defined above. In particularly interesting embodiments, the invention provides as a useful basis for a viable vaccine, the cell as defined above which contains a gene coding for the hydrolytically active enzyme whose expression is stochastically induced.

In useful embodiments of the invention, the cell which contains the DNA sequence coding for an immunologically active gene product further comprises means for transporting the epitope, when expressed, to the outer surface of the cell, i.e. translocating it across the cell membrane. Preferably such a translocation is obtained by inserting the gene coding for the epitope into a nucleotide sequence coding for an outer cell surface polypeptide structure such as fimbriae which contains the fimbrillin protein, pili, flagellae or certain other surface proteins including as an example the OM protein found in *Streptococcus* species. By providing the cell with such a hybrid nucleotide sequence being expressible in the cell, the gene product hereof will be a fusion or hybrid protein comprising the epitope and the relevant cell surface structure.

A cell in which a fusion protein is expressed which comprises the epitope fused to a surface structure protein by which the cell can adhere to the mucosal cells of a body to which the cell is administered is considered to be particularly useful in that the epitope will become in close contact with the mucosa and thereby effectively stimulate a protective immune response in the form of the excretion of secretory antibodies of the IgA and IgG classes.

Furthermore, the adhesion of the epitope-carrying cell will ensure that the cell is retained in the human or animal body for a period of time which is sufficient to obtain the desired immune response. It is considered that a satisfactory immunization typically may be obtained if the cell is present in sufficient numbers in a particular body environment such as the intestinal tract for a period in the range of 15-30 days, depending on the nature and the activity of the epitope expressed from the cell.

As it will be understood from the above description of the gene coding for the cell function-limiting hydrolytically active enzyme and the regulatory nucleotide, the present invention may provide useful means of providing live vaccines based on recombinant organisms which are immunologically effective and which can be used without the risk of undesired spreading of recombinant genes to the microflora of humans and animals or to the outer environment.

In accordance with the invention, a useful cell for the preparation of a live vaccine is one selected from a bacterial species which inherently contains an outer surface structure as mentioned above. Such species include as examples species of *Enterobacteriaceae* such as *Salmonella* and *E. coli* species, *Vibrionaceae* and *Pseudomonadaceae*. It will be understood that strains of such species which are particularly useful in the present invention as the basis of a live vaccine as defined above, are non-pathogenic strains or strains having a low pathogenicity.

The epitope expressed by a cell as defined above may be an epitope derived from any pathogenic organism or agent the obtainment of immunity against which is desirably. Such pathogens include viruses, bacteria and eucaryotic organisms such as fungi, yeast or protozoa.

In commercially important embodiments, the cell may contain a nucleotide sequence coding for a pesticidally active gene product. In this context, the term "pesticidally active gene product" is used to denote a product which when expressed in a cell being released to an environment where there is a need to reduce or eliminate the presence of pests including insect pests, vermins such as rodents or birds. Such pests may be controlled by the administration of toxic chemical pesticides to the infestated environment, but recently various naturally occurring pesticidally active organisms including viruses, bacteria and fungi have been used as biological pest control products.

Prominent examples of such pesticidally active organisms include biotypes or strains of the species *Bacillus thuringiensis* which produce crystalline proteins being toxic to insects, in particular to caterpillars, and several viruses being pathogenic for insects in the larval stage or in the adult stage. However, the pesticidal effect of such organisms is frequently less satisfactory and there is a strong need in farming, forestry and horticulture to provide improved pesticidally active organisms. One approach to solving this problem is to construct genetically engineered organisms having an increased toxic effect or a better survival rate in the environment.

Should such improved organisms be developed, their use in the environment will, as a consequence of current public concern of the potential risks involved in deliberate release of such toxic or pathogenic GEMs, only be approved by official environmental agencies if it can be demonstrated that the release

does not lead to an undesired propagation or to an extended survival of such organisms in the environment to which they are applied.

The present invention clearly provides the means of limiting the survival in the environment of genetically engineered pesticidally active organisms. As it has been explained above, the rate of expression of the cell function-limiting hydrolytically active enzyme may be regulated stochastically and thus the survival rate of pesticidally active cells may conveniently be adapted to any specific need. Also, the cell function-limiting effect may, in accordance with the present invention be adjusted by selecting a hydrolytically active enzyme which has an appropriate cell function-limiting effect.

In another useful embodiment, the invention provides a cell in which the DNA sequence not naturally related to the gene coding for the hydrolytically active enzyme, is a sequence coding for a pollutant-degrading gene product. It is known that several xenobiotic compounds polluting the outer environment including soil and water can be degraded by microorganisms having an inherent capability of degrading these compounds. Obviously, the technology of genetic engineering provides means of providing improved organisms having an increased pollutant-degrading capacity or having the capacity to degrade a broad range of compounds, in particular hydrocarbons.

However, the public concern as mentioned above are also relevant in this context and accordingly, the present invention provides useful means of providing improved pollutant-degrading cells, the survival of which can be controlled by regulating the expression of the cell function-limiting hydrolytically active enzyme as it is defined above. In particularly preferred embodiments, the cell contains a gene coding for a pollutant-degrading gene product, the expression

of which is induced by the presence of a pollutant degradable by the cell.

Cell as defined herein which is a transformed cell

In a further specific embodiment of the invention, the cell according to the invention and as defined above, is a cell which is transformed with a recombinant replicon or recombinant replicons containing a gene whose expression results in the formation of the cell function-limiting enzyme, the expression of said gene being regulated by a regulatory nucleotide sequence which is contained in the recombinant replicon containing the gene or in another recombinant replicon present in the transformed cell.

Replicon containing a gene coding for the intracellularly hydrolytically active cell function-limiting enzyme and optionally a regulatory nucleotide sequence

As mentioned above, the present invention pertains in a further aspect to a recombinant replicon containing the regulatably expressible gene encoding an intracellularly active hydrolytic enzyme as defined above, having, when it is expressed in a cell, a function-limiting effect thereon, said gene being regulated by a regulatory nucleotide sequence as also defined above and which sequence is operably linked to the expressible gene. In accordance with the invention, the regulatory nucleotide sequence may be a sequence contained in the replicon or in another replicon present in the cell containing the expressible gene.

The replicon may in accordance with the invention also be a replicon wherein the gene coding for the hydrolytically active enzyme and/or the nucleotide sequence regulating the expression of said gene, when such a sequence is present in the replicon, is mutated at one or more sites, so that the cell function-limiting effect of the enzyme encoded by the gene, when it is expressed in a cell containing the replicon,

is the same or increased relative to the cell function-limiting effect of the enzyme as expressed in a cell containing a replicon as defined herein not having been mutated.

In one embodiment, the replicon may comprise a further regulatably expressible gene which encodes a non-enzyme cell function-limiting function, the expression of which may be regulated by a regulatory nucleotide sequence of the same type as the sequence regulating the gene coding for the cell function-limiting hydrolytically active enzyme. In other embodiments, the nucleotide sequence regulating the expression of the non-enzyme cell function-limiting function is of a different type which however, may also be capable of regulating the expression of the gene encoding the cell function-limiting hydrolytically active enzyme.

Said further regulatably expressible gene may in useful embodiments be the *hok* gene from the *parB* region of plasmid R1 or a DNA sequence which is functionally homologous to the *hok* gene or the *E. coli* chromosomal *gef* gene.

In other useful embodiments, the replicon may further comprise a DNA sequence as defined above which is not naturally related to the replicon and which encodes a gene product selected from an immunologically active gene product, a pesticidally active gene product and a pollutant-degrading gene product.

<u>Population of cells containing a gene coding for an intracellularly hydrolytically active exoenzyme having cell function-limiting effect</u>

As mentioned above, the present invention pertains in a further aspect to a population of cells consisting of a multiplicity of cells as they have been defined above. The cell population may preferably comprise cell having been transformed with a recombinant replicon as defined herein which is capable of replicating in the cells. Since the cell

population in interesting uses may be released to the outer environment, the cells may in advantageous embodiments be bacterial cells which are selected from species whose natural habitat is a habitat selected from soil, surface water and plants, such as gram-negative bacterial species.

In this context, it is interesting to note that the survival of a population of cells as presently defined and in which the expression of the gene coding for the cell function-limiting enzyme is regulatable by a repressor substance which is present in the cells, but in different amounts depending on the physiological state of the cells, may, when such a population is applied to a human or an animal body, or to the outer environment, be regulated as a result of a decay of the repressor substance to an extent whereby the repressor substance is converted to a non-functional form. Provided the amount of the repressor substance in individual cells of a population being released, is different, it is assumed that this decay will result in a gradually increasing loss of viability of the cells of the population.

Method of limiting the survival of a cell population

As it has also been mentioned above, the present invention provides in one aspect a method of limiting the survival of a cell population in a first or a second environment wherein the regulatory nucleotide sequence is regulatable by an environmental factor as defined herein. The method may in preferred embodiments be related to a cell population as defined above.

In specific embodiments of this method, the survival of the cell population is limited in a first environment in which the gene coding for the cell function-limiting hydrolytically active enzyme is expressed whereby the cell population is contained in the first environment. In the present context, a first environment is typically the place of primary propagation of the cell such as a fermentation vessel.

In another specific embodiment of the method, the survival of the cell population is limited in a second environment which may be a first environment changing to a chemically different second environment, e.g. by the depletion of certain chemicals or the addition of such chemicals, or to a physically different second environment which change e.g. may take place by a shift in temperature or by a change of light intensity.

As it has been mentioned above, a cell population as defined herein may also by a cell population wherein the expression of the gene coding for the function-limiting enzyme is regulatable by a repressor substance which can undergo a decay or a degradation when said cells are released to a human or animal body or the outer environment to an extent whereby the repressor substance is converted to a non-functional form, said repressor substance being present in the cells of the population in different amounts whereby as a result of said decay the function of the cells of the population will gradually be limited.

Accordingly, the present invention provides in one useful embodiment a method of limiting the survival of a cell population by providing the cells with a gene coding for the hydrolytically active enzyme which is operably linked to a regulatory nucleotide sequence encoding a repressor substance which can undergo a decay when said cells are released to a human or animal body or the outer environment, to an extent whereby the repressor substance is converted to a non-functional form, said repressor substance being present in the cells of the population in different amounts whereby as a result of said decay the function of the cells of the population will be gradually be limited.

A method of containing an extrachromosomal recombinant replicon

As defined above, the invention relates in one aspect to a method of containing an extrachromosomal recombinant replicon to a first kind of cells where the replicon is naturally transferable to a second kind of cells, in which method the recombinant replicon is preferably a recombinant replicon as defined herein. In one preferred embodiment, this replicon is one not containing a regulatory nucleotide sequence and advantageously, such a replicon contains a gene coding for a hydrolytically active enzyme which is an endonuclease capable of hydrolysing diesterphosphate bonds in nucleic acids in the first kind of cells containing the replicon to be contained.

Method of stochastically limiting the function of cells in a cell population

In a particularly interesting aspect, the invention pertains, as it has been mentioned above, to a method of stochastically limiting the survival of a cell population such as a cell population as defined above, said method comprising the transformation of the cells with a recombinant replicon as also defined herein. In one useful embodiment, the method comprises a method wherein the expression of the cell function-limiting hydrolytically active enzyme is induced as a result of a site-specific recombinational excision of an excisable negatively functioning regulatory nucleotide sequence which, while present in the cells, inhibits expression of said gene, the negatively functioning regulatory nucleotide sequence being contained in the recombinant replicon or in another recombinant replicon present in the cells.

In another useful embodiment of this method, the survival of the cell population transformed with a recombinant replicon as presently defined, is being limited as a result of the above-defined recombinational inversion of an invertible promoter sequence of the negatively functioning regulatory

sequence. In accordance with the invention, the negatively functioning regulatory sequence may be one contained in the recombinant replicon or it may be one present on another replicon present in the cells. As one suitable example, the promoter sequence may be a sequence carrying the fimA promoter or a functional homologue thereof.

An immunologically active composition

The immunologically active composition as defined above may in addition to the viable function-limited cell population comprise pharmaceutically acceptable carriers and additives. Such acceptable carriers include any vehicle which is used conventionally in vaccine production such as e.g. saline. In the present context, suitable additives include immune response-enhancing substances including as examples Freund's incomplete and complete adjuvant and other non-specific immunostimulating substances.

It may be preferred to provide the composition in the form of lyophilized compositions, optionally in combination with a suitable aqueous vehicle such as saline. The immunologically active composition as presently defined may contain different types of cells, each of which encoding a specific epitope. Alternative, the immunologically active composition may in accordance with the invention contain a population of cells containing a multiplicity of genes coding for an immunologically active gene product, each gene coding for a different gene product.

The immunologically active compositions provided herein are useful vaccines for the immunization of both humans and animals.

In preferred embodiments, the immunologically active composition contains cells containing a sequence encoding for an immunologically active gene product which is a sequence coding for a fusion protein comprising said gene product and

a polypeptide, the presence of which results in the transportation of the fusion protein to the outer surface of the cells. As suitable examples, the composition may contain cells wherein the polypeptide is one selected from a polypeptide selected from a bacterial fimbrillin protein, a bacterial pilus, a bacterial flagellum and a bacterial OM surface protein.

Nucleotide sequences coding for such a polypeptide may conveniently be isolated from a bacterium selected from *Enterobacteriaceae*, *Vibrionaceae* and *Pseudomonadaceae*.

A pesticidally active composition

A pesticidally active composition as defined herein may in accordance with the invention, in specific useful embodiments contain a further DNA sequence encode a gene product which is toxic for insects or their progeny. In one interesting embodiment, the further DNA sequence is one derived from a strain of *Bacillus thuringiensis* which codes for an insecticidal protein.

As it has been explained above, cells as defined herein may code for a variety of pesticidally active gene products including the insecticidal protein mentioned above. Accordingly, this composition may be useful in controlling a wide range of pests and vermins. Cells which are useful in the pesticidally active composition may suitably contain a gene coding for the active gene product which are isolated from naturally occurring organisms producing or having a pesticidal activity.

The pesticidally active gene products coded for by the cells of the composition may be gene products which have a toxic effect on a pest or vermin, or the gene products may be a pest pathogenic virus which are expressed in the cells of the composition. The cells which in accordance with the present invention may be used in this composition include cells in

which the gene coding for a desired pesticidally active gene product can be expressed. Such cells include bacterial and fungal cells and plant and animal cells, optionally grown as cell cultures.

The pesticidally active composition may further comprise a suitable carrier. In this context, the term "suitable carrier" is used to indicate that the carrier comprises compounds which enhance the spreading of the composition in the pest infestated environment such as bulking agents which do not limit the survival of the cells in the composition, and compounds which may ensure the maintenance of the viability of the cells during production and storage of the composition and optionally also after application to the environment. As an example, a carrier may contain a compound which protect against the ultraviolet light which is detrimental to many organisms.

An environmental pollutant-degrading composition

The environmental pollutant-degrading composition as defined herein may preferably contain cells wherein the gene coding for the cell function-limiting hydrolytically active enzyme is only expressed when the pollutant degradable by the pollutant-degrading gene product is substantially degraded. As an example, such a composition may contain cells in which the expression of the hydrolytically active enzyme is repressed in the presence of the degradable pollutant.

DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the nucleotide sequence of the *Staphylococcus aureus* (Foggi) nuclease including the signal peptide encoding sequence, and the amino acid sequence of the signal peptide and the mature enzyme (SEQ ID NO:5, SEQ ID NO:6),

Fig. 2 shows the construction of pSNUC24-26 by ligation of PCR amplified, truncated *Staphylococcus aureus* (Foggi) nuclease encoding gene fragment digested with *Bam*HI and *Sac*I (deletion of 9 amino acid residues of the mature nuclease protein) to pET24d(+) also digested with *Bam*HI and *Sac*I,

Fig. 3 shows the nucleotide sequence of the mutant staphylococcal nuclease gene in pSNUC24-26 (SEQ ID NO:7). Amino acids in boxes represent essential amino acids required for binding to Ca^{2+} providing the conformational stability of the protein. Amino acids which are doubly underlined are important for conformational stability of the protein. A * indicates amino acid residues after mutation. Amino acid positions in brackets represent the position of the amino acids in the wild type nuclease gene without signal sequence (mature protein),

Fig. 4 shows kinetics of staphylococcus nuclease (SNUC) induction in *E. coli* HMS174(DE3) harbouring pSNUC24-26,

Fig. 5 illustrates intracellular stability of the mutant staphylococcal nuclease encoded by pSNUC24-26,

Fig. 6 shows kinetics of staphylococcus nuclease (SNUC) induction in *E. coli* NM522 harbouring pSNUC420-26, and

Fig. 7 illustrates intracellular stability of the mutant staphylococcal nuclease encoded by pSNUC420-26.

EXAMPLES

The below reference examples 1 and 2 corresponds to Examples 13 and 14, respectively in the co-pending application WO 93/20211 and are included herein for reference purposes. In these reference examples indications of figures refer to figures in the above co-pending application.

REFERENCE EXAMPLE 1

Intracellular expression and development of a conditional cell function-limiting system in *Escherichia coli* using a nuclease gene from *Staphylococcus aureus*

A. Bacterial strains, plasmids and oligonucleotides used

E. coli JM109 was purchased from Invitrogen Inc., San Diego, CA. Plasmid pFOG 408 containing the nuclease gene of *Staphylococcus aureus* Foggi strain was a gift from A. Meeker. The cloning vector plasmid pUHE24-2 was obtained from S. Molin. The oligonucleotides for PCR amplification were custom-synthesized by GENOSYS Inc., USA.

B. PCR amplification of the nuclease gene

The nuclease gene from the plasmid pFOG 408 without its signal sequence was PCR amplified using two oligonucleotide primers L-SNUC and R-SNUC. The primer L-SNUC (SEQ ID NO:1) was 37 nucleotides long (5'GATCCGGATCCGCAACTTCAAC-TAAAAAATTACATAA-3') with a 11 nucleotide overhang at the 5'-end consisting of *Bam*HI, *Acc*II, and *Hpa*II restriction endonuclease sites. The primer R-SNUC (SEQ ID NO:2) was 33 nucleotides long (5'-GGTACCGGAATTCGTGCCACTAGCAGCAGTGAC-3') with *Eco*RI, *Hpa*I, and *Kpn*I restriction endonuclease sites. PCR amplifications were performed in a DNA thermal cycler 480 (Perkin Elmer Cetus) using Ampli*Taq* DNA polymerase (2.5 units) (Perkin Elmer Cetus), 200 μM of each of the dNTPs (Pharmacia), PCR reaction buffer (10X reaction buffer contained 500 mM Tris.HCl [pH 8.9], 500 mM KCl, and 25 mM $MGCl_2$), and 0.5 μM of each of the primers. The PCR amplification was performed for a total of 25 cycles, each cycle consisting of 1 min denaturation at 94°C, 1 min primer annealing of 55°C, and 1 min primer extension at 72°C. Successful PCR amplification of a 0.507 kb DNA fragment was determined

by running 1% Seakem agarose gel (FMC Bioproducts), stained with EtBr, and visualized under a UV transilluminator.

PCR amplification using the primers L-SNUC and R-SNUC produced a single DNA band of 0.507 kb in size indicating no non-specific priming. The upstream primer L-SNUC was designed precisely so that it would prime the first nucleotide of the first amino acid residue after the signal sequence of the gene (cf. the sequence defined above). Moreover, a 11 bp overhang at the 5'-end of the primer ensured the synthesis of the gene from the desired nucleotide; thus eliminating the possibility of frameshift mutation during PCR amplification. The downstream primer R-SNUC was located further downstream of the stop codon and the possible hairpin structure outside the coding sequence of the gene, ensuring that all the amino acid including the stop codon of the gene remain intact and functional.

C. Cloning of the amplified nuclease gene

The amplified DNA of the staphylococcal nuclease gene without its signal sequence was end-repaired with DNA *polI* Klenow fragment to create blunt ends as described by Ausubel et al (1987). The end-repaired blunt ended amplified nuclease gene was purified by using a Centricon 100 microconcentrator (Amicon, MA). pUHE24-2 was linearized with *Bam*H1, end-repaired to create blunt-ends and purified by following the procedures described by Ausubel et al (1987). Cloning and transformation of the cloned DNA into *E. coli* JM109 was performed as described by Ausubel et al (1987). The transformed colonies were screened for the appropriate clones by replica plating on DNAse test agar (Difco) plates containing methyl green as indicator dye, 40 µg ampicillin per ml, 1 mM Isopropyl-ß-D-thiogalactopyranoside (IPTG), 10 mM $CaCl_2$, and 1 mM $MgCl_2$.

Colonies which produced indicator colour were further tested for clones by restriction analysis, PCR amplification, and

DNA sequence analysis. Plasmid DNAs from the putative clones, designated as pSNUC-1 and pSNUC-3 were isolated and purified by alkaline lysis method as described by Ausubel et al (1987). The isolated plasmid DNAs were digested with *Hin*dIII restriction enzyme (US Biochemicals) according to the manufacturer. The restriction enzyme digested plasmid DNAs were analyzed by 10% polyacrylamide gel electrophoresis, stained with EtBr, and visualized by a UV transilluminator. The nucleotide sequence analysis of the cloned fragments in both plasmids, pSNUC-1 and pSNUC-3, were determined by Sanger dideoxy method (Sanger et al, 1977) using a Sequenase sequencing kit (US Biochemicals) and the L-SNUC primer. These two clones were further characterized for induction and expression of the nuclease. Also they were tested for their ability to grow on LB agar plates containing ampicillin (40 µg per ml), 10 mM $CaCl_2$, 1 mM $MgCl_2$, with or without 1 mM IPTG.

The transformed cells were initially selected on LB agar plates containing 40 µg per ml ampicillin. Two putative clones which showed indicator colour change on DNAse test agar plate, were further tested on LB agar containing ampicillin, $CaCl_2$, $MgCl_2$, and IPTG. *E. coli* JM109 containing pSNUC-1 showed no growth on agar plate containing IPTG even after incubation up to 72 hours (Fig. 11). In contrast to this, *E. coli* JM109 carrying pSNUC-3 showed inhibited growth on similar type of agar plate containing IPTG (Fig. 12). In both cases, the control LB agar plates containing $CaCl_2$ and $MgCl_2$, but no IPTG showed complete growth within an 18-24 hours' time period. In this experiment, the inoculum sizes were kept as equal as possible for both types of plates, and they showed consistent results every time when repeated for a total of 7 times.

Restriction analysis of the plasmids pSNUC-1 and pSNUC-3 with *Hin*dIII produced expected DNA bands of approximately 0.219 kb and 4.0 kb bands (Fig. 13). Since there was one *Hin*dIII restriction site within the coding sequence of the nuclease

gene and one on the vector, it was possible to determine the right orientation of the nuclease gene on the vector.

Nucleotide sequence analysis of the first 40 bases of the cloned fragments in both pSNUC-1 and pSNUC-3 plasmids at their 5'-ends confirmed the presence of the nuclease gene in the right orientation.

D. Induction of pSNUC-1 and pSNUC-3

The *E. coli* JM109 (pSNUC-1) and *E. coli* JM109 (pSNUC-3) were grown in LB broth containing 10 mM $CaCl_2$ and 1 mM $MgCl_2$ at 37°C for 1 hour with shaking at 100 rpm till the optical density (OD_{450}) reached 0.5-0.7 when 1 mM sterile IPTG solution was added for induction. Before and after the addition of IPTG the OD_{450} was recorded every hour, a fraction of the cultures was serially diluted in phosphate buffer (pH 7.2) and plated on LB agar plates with 10 mM $CaCl_2$ and 1 mM $MgCl_2$ to determine viable plate counts. A control culture of each of the plasmid constructs was kept to compare the expression and killing efficiencies of the nuclease gene.

E. Expression of the cloned nuclease gene following induction

E. coli JM109 (pSNUC-1) and *E. coli* JM109 (pSNUC-3) were grown in LB broth as described earlier to mid-exponential phase (OD_{450} of 0.4 to 0.5) and IPTG was added for induction. The induction was carried on for 20 min when the cells were centrifuged and prepared for the total protein analysis by SDS-PAGE as described by Ausubel et al (1987) using the Mini-Protean II gel system (BioRad). The PAGE separated proteins were stained with commassie blue. Uninduced cultures of *E. coli* JM108 (pSNUC-1) and (pSNUC-3), cultures of *E. coli* AR120 (pFOG408), and *E. coli* JM109 (pUHE24-2) were used for controls.

For further characterization of the cloned nuclease genes in pSNUC-1 and pSNUC-3, a western blot was performed using

rabbit antibody (a gift from A. Meeker). The proteins from the PAGE were transferred to nitrocellulose membrane (Millipore) using the Mini-Trans Blot system (BioRad). A 1:15,000 dilution of rabbit antibody was used and detected with biotinylated goat antirabbit immunoglobulin and streptavidin peroxidase (Fisher Biotech). The color development was performed by incubating in the peroxidase substrate by 4-chloro-1-naphthol. Appropriate controls were tested in this experiment.

Induction of *E. coli* JM109 (pSNUC-1) with IPTG showed significant decline in cell numbers between 2-7 hours' time period as determined by the OD_{450} reading and viable plate counts (Figs. 14a and 14b). The total cell number for *E. coli* JM109 (pSNUC-1) per ml declined form $2x10^6$ to $1.5x10^4$ between 0-7 hours' time period as determined by viable plate counts. In contrast to this, when *E. coli* JM109 (pSNUC-3) was induced with IPTG, a slow increase in cell number was evidenced as compared to the uninduced control as determined by the OD_{450} measurements and viable plate counts (Figs. 15a and 15b). The total cell number increased form $3x10^6$ to $7x10^8$ per ml between 0-7 hours as compared to the uninduced control which was $3x10^6$ to $2x10^{10}$ within the same time period. From these results it can be predicted that the lower killing efficiency of the plasmid pSNUC-3 upon induction as compared to pSNUC-1 may be due to mutation generated by misincorporation of nucleotide(s) during the PCR DNA amplification process. However, it is not obvious which of the two clones, if not both, has the altered nucleotide sequence.

The phenotypic expression of the cloned nuclease genes in pSNUC-1 and pSNUC-3 plasmids on the DNAse test agar containing methyl green indicator dye showed expected color change. The pSNUC-1 showed more rapid color change than pSNUC-3. The control plasmid vector pSM1128 did not show any change in color on the same agar plates, whereas, plasmid pFOG408 showed significant color change.

The PAGE gel for total protein analysis showed faint bands of approximately 17.8 kD size range for plasmids pSNUC-1 and pSNUC-3 following induction with IPTG. The uninduced controls and pUHE24-2 did not show any such band (Fig. 16). Since nuclease is an extracellular secretory protein, the *E. coli* strain carrying pFOG408 plasmid showed a very faint band in the gel. To confirm the expression of the cloned nuclease gene in plasmids pSNUC-1 and pSNUC-3, a western blot was performed. In the western blot, distinct protein bands of approximately 17.8 kD were evidenced from the induced *E. coli* JM109 strains carrying pSNUC-1 and pSNUC-3 plasmids, and *E. coli* AR120 strain carrying pFOG408 plasmid (Fig. 17). The uninduced cultures and control plasmid pSM1128 did not show any band. Another non-specific band of approximately 20 kD size was noticed in all samples, which seems to be the non-specific antigenic reaction against the secondary antibody. This experiment suggested that the nuclease was produced by both plasmids pSNUC-1 and pSNUC-3 upon induction. The lower killing efficiency for the pSNUC-3 plasmid could be due to the altered nucleotide sequence which has changed the protein structure and its functions for the substrate. Alternatively, this could be the case for the plasmid pSNUC-1 for its relatively higher killing efficiency. Complete nucleotide sequence analysis for both clones may resolve this question and also provide the information on possible sites where change of nucleotide sequence may make this enzyme more effective.

REFERENCE EXAMPLE 2

Determination of intracellular stability of cloned staphylococcal nuclease in *Escherichia coli* by ELISA assay

The staphylococcal nuclease gene *snuc* without its signal sequence was PCR-amplified and cloned in *Escherichia coli* JM109 as described in Example 13 using the expression vector pUHE24-2, in the *Bam*HI restriction site (pSNUC-1 and pSNUC-3). The nuclease was expressed intracellularly when induced with IPTG. The stability of the intracellularly expressed SNUC enzyme in *E. coli* following induction was determined by ELISA assay using antibodies raised in rabbits. Two approaches were pursued to determine the stability of the intracellularly expressed SNUC enzyme:

1. Transcriptional inhibition following induction by blocking mRNA synthesis with rifampicin.

2. Removal of inducing agent (IPTG) from the culture following induction.

1. Transcriptional inhibition following induction by blocking mRNA synthesis with rifampicin

E. coli cells harbouring pSNUC-1 or pSNUC-3 were grown in liquid broth at 37°C till the optical density (OD_{450}) was between 0.12 and 0.15 (early exponential growth phase). The cells were then induced with IPTG for 30 minutes, and rifampicin was added to the culture to block transcription. The incubation was carried on for the next 3 hours. *E. coli* JM109 without pSNUC-1 or pSNUC-3 was used as the control.

Aliquots of 1 ml of the cultures were collected before induction, 30 minutes after induction, and every 30 minutes after the addition of rifampicin. The cells were centrifuged, washed twice with phosphate buffer (pH 7.2), and disrupted by

sonication to release the total cellular proteins. The sonication was carried on (8 to 10 minutes with a pulse for 30 seconds at every 30 seconds interval) till the sample became clear. The debris was spun down and the supernatant (lysate) was used for ELISA assay.

An aliquot of each of the samples was subjected to spectrophotometric measurement to determine the total protein concentration. ELISA assay was performed using equal quantity of total cellular protein from each lysate. Typically, 10 to 55 µl of lysates (depending on the concentration of each lysate) from various samples were absorbed in a microtiter plate (Corning) for about 16 hours (overnight) at 4°C. The wells were then washed 5 times with phosphate buffer (pH 7.2). The unreacted surface of the microtiter plate was blocked with 2% bovine serum albumin (BSA) from Sigma Chemical at room temperature for 1 hour with gentle shaking. The excess BSA from the microtiter plate was washed off 5 times with phosphate buffer (pH 7.2). 1:15.000 fold diluted polyclonal primary antibodies raised against the purified extracellular staphylococcal nuclease in rabbits, was reacted with preabsorbed total cellular proteins for about 16 hours (overnight) at 4°C. The unreacted primary antibodies were washed off 5 times with phosphate buffer (pH 7.2).

The bound primary antibodies were then treated with secondary antibodies, goat antirabbit-Ig-biotin (CloneTech) for 1 hour at room temperature with gentle shaking. After washing off excess secondary antibodies, streptavidin-horseradish peroxidase (HRP-SA) conjugate was added to the microtiter plate and conjugated with the secondary antibodies. After washing off excess HRP-SA, colour development was carried out using 0.1 ml of substrate [2,2'-azinobis (3-ethyl benzthiazoline sulphonic acid) in a 0.1 M citrate buffer, pH 4.2 with 0.03% hydrogen peroxide]. The absorbance was read at 415 nm in an ELISA microtiter plate reader (Flow Laboratories, Inc., McClean, Virginia).

2. Removal of inducible agent (IPTG) from the culture following a short induction stage

E. coli cells harbouring pSNUC-1 or pSNUC-3 were grown to early exponential phase (OD_{450} of 0.12 to 0.15) when 1 mM IPTG (final concentration) was added to the culture. The induction with IPTG was carried on for 5 minutes at 37°C and the cells were centrifuged and washed twice with phosphate buffer (pH 7.2). The cells were resuspended in broth and incubated at 37°C for another 3 hours. Aliquots of 1 ml each of the cultures were collected before induction and 5 minutes after induction. Also, aliquots were collected by centrifugation, washed twice with phosphate buffer (pH 7.2) and sonicated to release total cellular proteins. Cultures of *E. coli* JM109 without pSNUC-1 or pSNUC-3 was used as the negative control.

Total cellular proteins were released by disrupting the cells using a sonic disruptor as described above. ELISA assays for intracellularly expressed staphylococcal nuclease was also performed as described above.

The amount of staphylococcal nuclease protein encoded by pSNUC-1 and pSNUC-3 after transcriptional inhibition as defined above, was found to be significant amounts following induction with IPTG. The data for pSNUC-1 and pSNUC-3 are summarized in Tables 5 and 6, respectively. The expression of staphylococcal nuclease increased about 2-fold during the first 30 minutes after induction as compared with the amount detected prior to induction. The activity of the nuclease enzyme remained during the first 2 hours following rifampicin treatment. Between 2 and 3 hours after addition of rifampicin (2.5 and 3.5 hours after induction with IPTG) the activity of the nuclease was dropped significantly. From this experiment it can be concluded that the staphylococcal nuclease is stable intracellularly for at least 1.5 hours after the inhibition of transcription.

Table 5. Determination of the stability of intracellularly expressed staphylococcal nuclease expressed by pSNUC-1 following induction with IPTG and treatment with rifampicin

Treatment	Time (h)	ELISA readings
	0	0.427
IPTG	0.5	0.584
Rifampicin	1.0	0.666
	1.5	0.685
	2.0	0.675
	2.5	0.542
	3.0	0.488
	3.5	0.407
Negative control	1.0	0.276

Table 6. Determination of the stability of intracellularly expressed staphylococcal nuclease expressed by pSNUC-3 following induction with IPTG and treatment with rifampicin

Treatment	Time (h)	ELISA readings
	0	0.399
IPTG	0.5	0.495
Rifampicin	1.0	0.557
	1.5	0.593
	2.0	0.555
	2.5	0.443
	3.0	0.427
	3.5	0.378
Negative control	1.0	0.258

After induction of with IPTG for a short period of time (5 minutes), the cells were centrifuged, washed with phosphate buffer (pH 7.2), and grown in a rich medium for 150 minutes to determine the stability of the SNUC in the cells.

The summary of the data is presented in Tables 7 and 8. There was at least 3-fold increase in the amount of SNUC following induction. The SNUC protein was found to be stable in pSNUC-1 for 1.5 to 2 hours (between 5 and 125 minutes) after induction and 1 to 1.5 hours (between 5 and 95 minutes) in pSNUC-3 as it was determined by the ELISA assay. After 125 minutes for pSNUC-1 and 95 minutes for pSNUC-3 following removal of the inducing agent (IPTG), the SNUC activity decreased significantly.

Table 7. Determination of the stability of intracellularly expressed staphylococcal nuclease expressed by pSNUC-1 following removal of IPTG after 5 minutes of induction

Treatment	Time (min)	ELISA readings
	0	0.270
IPTG		
	5	0.565
Removal of IPTG		
	35	0.560
	65	0.543
	95	0.501
	125	0.456
	155	0.329
Negative control	35	0.204

Table 8. Determination of the stability of intracellularly expressed staphylococcal nuclease expressed by pSNUC-3 following removal of IPTG after 5 minutes of induction

Treatment	Time (min)	ELISA readings
	0	0.171
IPTG		
	5	0.461
Removal of IPTG		
	35	0.458
	65	0.430
	95	0.375
	125	0.269
	155	0.210
Negative control	35	0.164

EXAMPLE 1

Intracellular expression and stability of a mutant *Staphylococcus aureus* nuclease gene in *Escherichia coli*

A. Cloning of the mutant staphylococcal nuclease gene for intracellular expression

In applicants' co-pending application US Serial No. 08/135,665 it is disclosed that the wild type *Staphylococcus aureus* (Foggi) nuclease gene isolated from the plasmid pFOG408, without its signal sequence may be expressed intracellularly and used as the basis for a conditional cell function-limiting system in bacteria. However, it has been found that although the expression of the wild type nuclease protein is predominantly intracellularly, some export of the signal peptide-less enzyme outside the cell may be observed. Accordingly, it was attempted to prevent such extracellular

transport of staphylococcal nuclease by removal, not only of the signal peptide, but of further amino acid residues of the mature protein.

DNA sequences encoding a total of 30 amino acid residues (21 amino acid residues of the signal peptide sequence and 9 amino acid residues of the mature protein, cf. Fig. 1) of the nuclease derived from the *Staphylococcus aureus* (Foggi) strain (ATCC 27355) were deleted and the rest of the gene segment constituting 426 bp was PCR amplified using oligonucleotide primers flanked with appropriate restriction enzyme sites and cloned into pET24d(+) plasmid vector (5307 bp) (Novagen, Inc. 597 Science Drive, Madison, WI 53711). The location, lengths and the nucleotide sequences of the oligonucleotide primers which were used for PCR amplification of the staphylococcal nuclease gene are described in Fig. 3. For the intracellular biological activity, the specific amino acid residue # 31 (Glu encoded by the nucleotide sequence GAA) at the N-terminal end of the nuclease gene was selected as the first amino acid of the gene. This selection of the first amino acid was based on Tucker et al., 1979, Molecular and Cellular Biochemistry, 23, 131-141, according to which the deletion of amino acid residues starting from # 10 and onwards of the *Staphylococcus aureus* (Foggi) nuclease produces a marked fall in both structure and activity of the enzyme.

For cloning, the pET24d(+) vector was treated with *Bam*HI and *Sac*I restriction endonucleases and ligated to similarly digested PCR amplified staphylococcal nuclease gene fragment as defined above. The pET24d(+) plasmid vector comprises a promoter sequence from bacteriophage T7, the *lac* operator (*lac*O) DNA segment which interacts with the repressor protein in the absence of IPTG, a DNA sequence for ribosome binding site (rbs), followed by several unique bacterial restriction endonuclease sites (multiple cloning sites or MCSs) for the insertion of a foreign DNA segment. The repressor protein is encoded by the *lac*I gene which is located on the pET24d(+)

vector. Also, the pET24d(+) vector comprises a gene that encodes for resistance to kanamycin (KM), and the colE1 replication origin. The DNA sequence (ATG) that codes for the translational start codon (Met) is located immediately after the rbs in the multiple cloning site for expression of the nuclease protein from the truncated nuclease gene segment without the translational first codon. There was no *Bam*HI or *Sac*I restriction endonuclease sites within the nuclease gene fragment.

The pET24d(+) vector ligated to the digested PCR amplified staphylococcal gene fragments as defined above was used for transformation of the *E. coli* strain HMS174(DE3) (genotype: F^-, *rec*A, r_{k12}^- , m_{k12}^-, Rif^r) (Novagen, Inc., Madison, WI) which carries the T7 RNA polymerase gene under the *lac*UV5 control located on the chromosome. Transformants were selected on DNAse agar with toluidine blue (BBL cat# 99081) supplemented with 75 μg per ml of kanamycin. A number of clones with the characteristic nuclease "halo" were identified after 16-24 h of incubation at 37°C. However, a few colonies were found to be relatively slow growing on the same agar plates and showed secondary nuclease activity as evidenced by a relatively slowly developing "halo" on the DNAse test agar. One of these relatively slow growing colonies was selected and further tested with regard to the nature of the nuclease gene. The cloned nuclease gene in the pET24d(+) which was isolated from the slow growing *E. coli* HMS174(DE3) transformants was designated as pSNUC24-26 (Fig. 2). pSNUC24-26 in the *E. coli* HMS174(DE3) host strain was deposited in accordance with the Budapest Treaty with the American Type Culture Collection (ATCC, 12301 Parklawn Drive, Rockville, MD 20852, USA on 8 October 1993 and assigned the accession number ATCC 69462.

B. Determination of genetic characteristics of the staphylococcal nuclease gene cloned in pSNUC24-26

In order to determine the genetic characteristics of the nuclease gene in pSNUC24-26, nucleotide sequence analysis was performed by following the Sanger dideoxy chain termination method using the T7 promoter primer (SEQ ID NO:3) (5'-TAATACGACTCACTATA-3') and the T7 terminator primer 3'-TGGCGACTCGTTATTGATC-5'(this primer being complementary to the sequence 5'- ACCGCTGAGCAATAACTAG-3' (SEQ ID NO:4)). The complete nucleotide sequence of the nuclease gene in pSNUC24-26 is presented in Fig. 3. A number of point mutations and addition of several base pairs were detected in the cloned staphylococcal nuclease gene in pSNUC24-26 resulting in a mutant form of the gene. A small DNA sequence, 5'-GGATCG-3', was inserted by an unknown mechanism immediately upstream of the *Bam*HI site of the left side primer sequence during the PCR DNA amplification. Also, deletion of a nucleotide base, C, in the second position of the second codon [CCT > CT, i.e. C(ΔC)T], and transition of A > G at the 4th and 36th codons (ACT > GCT at the 4th and AAG > GAG at the 36th positions) were evidenced.

Although, the deletion of a nucleotide base, C, at the second codon resulted in a frameshift mutation, the wild type amino acid residues of the nuclease protein were restored at the 3rd codon (GCG - Ala) and from the 5th codon (TTA - Leu) onwards. As a result, for the conformational stability and biological activities, the amino acid residues at positions 19 (GAT - Asp), 21 (GAT - Asp), 40 (GAC - Asp), 41 (ACA - Thr) and 43 (GAA - Glu) that have been shown to be essential for binding to Ca^{2+} (Tucker et al., 1979, Molecular and Cellular Biochemistry, 23, 67-86) together with the amino acid residues at positions 35 (AGA - Arg), 84 (AAA - Lys), 85 (TAT - Tyr), 87 (CGT - Arg) and 113 (TAT - Tyr) that have been shown to be essential for intramolecular interactions to establish the "active site" of the protein (Tucker et al., 1979), were not affected by this mutation (cf. Fig. 3.

However, the changes in amino acid residues from Glu > Arg at position 10, from Pro > Thr at position 11 due to a single base deletion (CCT > CT) and the insertion of the DNA sequence 5'-GGATCG-3' upstream of the primer location, and changes in the amino acid residues of Thr > Ala at position 13 and of Lys > Arg at position 36 due to the point mutations (A > G) of the wild type nuclease gene may have altered the extracellular transport and relative biological activities of the protein encoded by the plasmid pSNUC24-26.

C. Kinetics of the pSNUC24-26-encoded staphylococcal nuclease in *E. coli* HMS174(DE3)

To determine the cell function-limiting (or killing) efficiencies of the mutant staphylococcal nuclease gene in the pSNUC24-26 plasmid, induction of the *E. coli* HMS174(DE3) carrying this plasmid was performed by the addition of 1 mM of IPTG in an exponentially growing liquid culture. Before and after addition of IPTG (i.e. induction), aliquots of serially diluted culture were tested on DNAse Test Agar plates without IPTG. A decrease in the cell number at the level of > 4 log was evidenced within the first 3 h time period following induction when plated onto DNAse Test Agar plates without IPTG (Fig. 4).

D. Intracellular stability of the mutant nuclease enzyme as determined by immunological assay

The stability of the mutant nuclease enzyme within the *E. coli* cells was determined by ELISA assay using polyclonal antibodies raised in rabbit against purified wild type *Staphylococcus aureus* (Foggi) nuclease. An exponentially growing culture of *E. coli* HMS174(DE3) carrying pSNUC24-26 was induced by the addition of 1 mM of IPTG for 30 minutes. Immediately after induction, 40 µg per ml of chloramphenicol was added to the culture to inhibit protein synthesis and the cells were continued to grow for another 3 hours. Aliquots of the culture were separated at every 30 min interval for up to

3 h, centrifuged, washed 2 x with phosphate buffered saline (PBS) (pH 7.2), resuspended in PBS and the cells were disrupted by using a sonic disruptor to release the total cellular proteins. An aliquot of the proteins released at every time period following chloramphenicol treatment was immobilized into a microtiter plate and ELISA assay was performed as described in reference example 2.

About 2.5 fold increase in the nuclease protein was evidenced following induction and the nuclease protein was stable within the cell > 1.5 h following chloramphenicol treatment (Fig. 5). Therefore, the intracellular stability of the mutant nuclease protein was not affected to the same extent as it was observed with the wild type nuclease protein. However, the basal level of production of nuclease was found to be extremely low in the mutant staphylococcal nuclease protein suggesting that the modifications in the nucleotide sequences may have contributed to less extracellular transport of the mutant protein.

E. Identification of the mutant nuclease protein by immunological assays

For identification of the mutant nuclease protein in the pSNUC24-26 plasmid, western blot analysis was performed by using polyclonal antibody raised against purified wild type *Staphylococcus aureus* (Foggi) nuclease in rabbit. Total cellular proteins in *E. coli* HMS174(DE3) harbouring pSNUC24-26 plasmid before and after IPTG induction were subjected to electrophoretic separation in denaturing polyacrylamide gel with appropriate size standards. The proteins were immobilized on a nitrocellulose membrane and subjected to western blot analysis according to the procedure as described in reference example 1.

The western blot analysis of the mutant staphylococcal nuclease showed very weak signal before induction. However,

following induction, the signal increased significantly. In the wild type clones, differences in the intensities of the nuclease enzyme before and after induction was indistinguishable. This suggests that the mutant nuclease gene encoded the modified protein that may significantly have prevented the extracellular transport of the modified mutant nuclease.

F. Cloning of the mutant staphylococcal nuclease gene in the pSE420 plasmid vector

The intracellular effectiveness of the above mutant staphylococcal nuclease (*snuc**) under the control of a different promoter element was tested by cloning the mutant staphylococcal gene with the upstream coding sequences under the control of the P_{tac} promoter/*lac*O (a synthetic promoter generated by the fusion of the tryptophan and the lactose promoters) in pSE420 (Invitrogen Corp., 3985 B Sorrento Valley Blvd., San Diego, CA 92121). The *Nco*I and the *Xho*I restriction endonuclease-digested fragment from pSNUC24-26 plasmid consisting of the mutant staphylococcal nuclease gene with the upstream coding sequences was purified and ligated to the similarly digested pSE420 plasmid vector and the resulting recombinant plasmid was designated pSNUC420-26.

pSNUC420-26 was transformed into *E. coli* NM522 (Genotype = F'{*pro*AB$^+$ *lac*I^q *lac*Z ΔM15}, *sup*E, *thi*1, (*lacpro*AB), *hsd*5 (r^- m^-) λ^- (Invitrogen Corp.)

pSNUC420-26 in the *E. coli* NM522 host strain was deposited in accordance with the Budapest Treaty with the American Type Culture Collection (ATCC, 12301 Parklawn Drive, Rockville, MD 20852, USA on 8 October 1993 and assigned the accession number ATCC 69463.

G. Kinetics of mutant SNUC induction in *E. coli* NM522 (pSNUC420-26)

The procedure for induction with IPTG and viable plate counts as described above for pSNUC24-26 was followed. The results of the induction are presented in Fig. 6. A significant reduction of cell numbers (>5 log) within the first 1.5 h time period was evidenced.

H. Stability of the mutant nuclease in *E. coli* NM522 (pSNUC420-26)

The intracellular stability of the mutant nuclease protein in pSE420-26 was determined by ELISA assay as described above for pSNUC24-26. The results are presented in Fig. 7. About 2-fold increase in the intracellular nuclease was evidenced following induction. Also, the intracellular stability of the mutant nuclease was determined to about 1.5 h following treatment with rifampicin. It was also observed that the basal level of nuclease expression during uninduced state was negligible. This result is comparable with that of the ELISA assay in pSNUC24-26.

I. Identification of the pSE420-26-encoded mutant nuclease by western blot analysis

Western blot analysis of the mutant staphylococcal nuclease in pSE420-26 showed low level of signal before induction and the higher signal following induction as observed above for the pSNUC24-26 plasmid.

EXAMPLE 2

The use of the *xth* exonuclease III gene from *Escherichia coli* in combination with a mutant staphylococcal nuclease gene (*snuc**) for the containment of genetically engineered micro-organisms

The exonuclease III of *E. coli* has multiple catalytic activities *in vivo*: (i) it has 3'-5'exonuclease activities specific for the double stranded DNA, (ii) it can remove a number of 3'-end termini from duplex DNA, including 3'-phosphates, (iii) it is an AP endonuclease which cleaves phosphodiester bonds at AP sites to yield base-free deoxyribose 5'-phosphate end groups, (iv) it has an RNase H activity which preferentially degrades the RNA strand in a RNA-DNA hybrid duplex (Weiss, 1981).

The exonuclease III of *E. coli* is encoded by the gene *xth*, the sequence of which has been determined and reported by Saparito et al. (1988).

The coding sequence including the ribosome binding site (SD sequence) of the *xth* gene was PCR amplified using two oligonucleotide primers L-XTH and R-XTH and cloned into the pCRII® vector and then cloned into the pSE420® plasmid vector (Novagen, Inc.) at the *Xba*I and *Kpn* restriction sites under the control of the *trp-lac* promoter (P_{tac})/*lac* operator (*lacO*) and $lacI^q$. The orientation of the cloned *xth* gene in pSE420 vector has been confirmed by restriction enzyme analysis. The P_{tac}/*lacO*::*xth* gene from the pSE420 vector will be recovered by restriction enzyme digestion and cloned into pUC18Not vector which already has the P_{tac}/*lacO*::*snuc** and the $lacI^q$ gene.

Following cloning, the P_{tac}/*lacO*::*xth*, P_{tac}/*lacO*::*snuc** and $lacI^q$ genes as a complete genetic cassette will be recovered from the pUC18Not vector as a *Not*I fragment and inserted into a similarly digested transposon vector pUTKM for insertion on

the chromosome of the host microbial strain. In this system, both the staphylococcal nuclease gene (*snuc**) and the exonuclease III (*xth*) gene can be expressed simply by addition of IPTG and the efficiency of killing of the host strain can be determined by viable cell plate counts. During the normal physiological conditions (uninduced stage), both genes will remain silent due to the binding of the repressor protein encoded by the *lacI* gene to the *lacO* DNA sequence.

EXAMPLE 3

The construction of a replicon simultaneously expressing the *snuc** and the *hok* genes

The P_{tac}/*lacO*::*snuc** and the $lacI^q$ gene cassette was recovered from the pSNUC26-420 vector using the *Eco*RV and *Xho*I restriction enzymes and blunt ended by "fill in" reaction. This fraction was cloned in pBAP24*h* vector (Bej et al., 1992) at the *Sal*I restriction site which was also blunt ended by "fill in" reaction. The pBAP24*h* vector has the *hok* gene under the control of the P_{tac}/*lacO* promoter-operator and the $lacI^q$. Upon induction with IPTG, both genes, *snuc** and *hok* can be expressed simultaneously. The cell function-limiting effects due to the simultaneous expression of the *hok* and the *snuc** genes can be monitored by induction with ITPG and viable cell plate counts. The clones with the appropriate orientation of the genes was confirmed by restriction analysis.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 60, line 33.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution

American Type Culture Collection (ATCC)

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville
MD 20852, USA

Date of deposit	Accession Number
8 October 1993	ATCC 69462

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [ ]

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Authorized officer: Anne Rydsing	Authorized officer

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(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 64, line 29.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution

American Type Culture Collection (ATCC)

Address of depositary institution (*including postal code and country*)

12301 Parklawn Drive
Rockville
MD 20852, USA

Date of deposit	Accession Number
8 October 1993	ATCC 69463

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) — This information is continued on an additional sheet [ ]

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

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Authorized officer

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Genexpress ApS
(B) STREET: Mothsvej 70
(C) CITY: Holte
(D) COUNTRY: Denmark
(E) POSTAL CODE (ZIP): 2840

(ii) TITLE OF INVENTION: Recombinant Staphylococcal Nucleases and Their Use in Limiting the Survival of Genetically Engineering Microorganisms

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATCCGGATC CGCAACTTCA ACTAAAAAAT TACATAA 37

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTACCGGAA TTCGTGCCAC TAGCAGCAGT GAC 33

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TAATACGACT CACTATA 17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACCGCTGAGC AATAACTAG 19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 763 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 215..727
(D) OTHER INFORMATION: /note= "The initiation codon GTG, while normally translated as Valine, may also be translated as N-formyl Methionine."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGTAACCGGC TAGTTGCGGC CGCTGCCAGC CATTTGCCAC TCTCCTTTTC ATCCGCATCG 60

GCAGGGTCAT CCGGGCGCAT CCACCACTCC TGATGCAGTA ATCCTACGGT GCGGAATGTG 120

GTGGCCTCGA AATTCTGTCA TAAAGTTGTC ACGGCCGAGA CTTATAGTCG CTTTGTTTTT 180

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ATTTTTTAAT GTATTTGTAC ATGGAGAAAA TAAA GTG AAA CAA ACG ACT ATT          232
                                       Met Lys Gln Thr Thr Ile
                                         1               5

GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAA GCC GCA          280
Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala
            10                  15                  20

ACT TCA ACT AAA AAA TTA CAT AAA GAA CCT GCG ACT TTA ATT AAA GCG          328
Thr Ser Thr Lys Lys Leu His Lys Glu Pro Ala Thr Leu Ile Lys Ala
        25                  30                  35

ATT GAT GGT GAT ACG GTT AAA TTA ATG TAC AAA GGT CAA CCA ATG ACA          376
Ile Asp Gly Asp Thr Val Lys Leu Met Tyr Lys Gly Gln Pro Met Thr
    40                  45                  50

TTC AGA CTA TTA TTG GTC GAC ACA CCT GAA ACA AAG CAT CCT AAA AAA          424
Phe Arg Leu Leu Leu Val Asp Thr Pro Glu Thr Lys His Pro Lys Lys
55                  60                  65                  70

GGT GTA GAG AAA TAT GGT CCT GAA GCA AGT GCA TTT ACC AAA AAA ATG          472
Gly Val Glu Lys Tyr Gly Pro Glu Ala Ser Ala Phe Thr Lys Lys Met
                75                  80                  85

GTA GAA AAT GCA AAG AAA ATT GAA GTC GAA TTC GAC AAA GGT CAA AGA          520
Val Glu Asn Ala Lys Lys Ile Glu Val Glu Phe Asp Lys Gly Gln Arg
            90                  95                  100

ACT GAT AAA TAT GGA CGT GGG CTA GCG TAT ATT TAT GCT GAT GGA AAA          568
Thr Asp Lys Tyr Gly Arg Gly Leu Ala Tyr Ile Tyr Ala Asp Gly Lys
        105                 110                 115

ATG GTA AAC GAA GCT TTA GTT CGT CAA GGC TTG GCT AAA GTT GCT TAT          616
Met Val Asn Glu Ala Leu Val Arg Gln Gly Leu Ala Lys Val Ala Tyr
    120                 125                 130

GTT TAC AAA CCT AAC AAT ACA CAT GAA CAA CAT TTA AGA AAA AGT GAA          664
Val Tyr Lys Pro Asn Asn Thr His Glu Gln His Leu Arg Lys Ser Glu
135                 140                 145                 150

GCA CAA GCG AAA AAA GAG AAA TTA AAT ATT TGG AGC GAA GAC AAC GCT          712
Ala Gln Ala Lys Lys Glu Lys Leu Asn Ile Trp Ser Glu Asp Asn Ala
                155                 160                 165

GAT TCA GGT CAA TAATGCTCAT TGTAAAAGTG TCACTGCTGC TAGTGGCAC               763
Asp Ser Gly Gln
            170
```

(2) INFORMATION FOR SEQ ID NO:6

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 170 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Met Lys Gln Thr Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr
 1               5                  10                  15

Pro Val Thr Lys Ala Ala Thr Ser Thr Lys Lys Leu His Lys Glu Pro
            20                  25                  30

Ala Thr Leu Ile Lys Ala Ile Asp Gly Asp Thr Val Lys Leu Met Tyr
        35                  40                  45

Lys Gly Gln Pro Met Thr Phe Arg Leu Leu Leu Val Asp Thr Pro Glu
    50                  55                  60

Thr Lys His Pro Lys Lys Gly Val Glu Lys Tyr Gly Pro Glu Ala Ser
65                  70                  75                  80

Ala Phe Thr Lys Lys Met Val Glu Asn Ala Lys Lys Ile Glu Val Glu
                85                  90                  95

Phe Asp Lys Gly Gln Arg Thr Asp Lys Tyr Gly Arg Gly Leu Ala Tyr
            100                 105                 110

Ile Tyr Ala Asp Gly Lys Met Val Asn Glu Ala Leu Val Arg Gln Gly
        115                 120                 125

Leu Ala Lys Val Ala Tyr Val Tyr Lys Pro Asn Asn Thr His Glu Gln
    130                 135                 140

His Leu Arg Lys Ser Glu Ala Gln Ala Lys Lys Glu Lys Leu Asn Ile
145                 150                 155                 160

Trp Ser Glu Asp Asn Ala Asp Ser Gly Gln
                165                 170
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 482 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATGGCTAGCA TGACTGGTGG ACAGCAAATG GGTCGGATCG GGATCCGAAC TGCGGCTTTA      60

ATTAAAGCGA TTGATGGTGA TACGGTTAAA TTAATGTACA AAGGTCAACC AATGACATTC     120

AGACTATTAT TGGTCGACAC ACCTGAAACA AGGCATCCTA AAAAAGGTGT AGAGAAATAT     180

GGTCCTGAAG CAAGTGCATT TACCAAAAAA ATGGTAGAAA ATGCAAAGAA AATTGAAGTC     240

GAATTCGACA AAGGTCAAAG AACTGATAAA TATGGAGCTG GGCTAGCGTA TATTTATGCT     300
```

GATGGAAAAA TGGTAAACGA AGCTTTAGTT CGTCAAGGCT TGGCTAAAGT TGCTTATGTT 360

TACAAACCTA ACAATACACA TGAACAACAT TTAAGAAAAA GTGAAGCACA AGCGAAAAAA 420

GAGAAATTAA ATATTTGGAG CGAAGACAAC GCTGATTCAG GTCAATAATG CGAGCTCGAG 480

CT 482

CLAIMS

1. A cell containing a gene whose expression results in the formation of a truncated and/or mutated *Staphylococcus aureus* nuclease which is present and hydrolytically active in the cytoplasm of said cell, the cell further containing a regulatory nucleotide sequence which regulates the expression of the gene, the expression of said gene leading to formation of the enzyme in the cell at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cell, to an extent whereby the function of the cell is being limited.

2. A cell according to claim 1 wherein the expression of the gene results in the formation of a nuclease being capable of hydrolysing diesterphosphate bonds in nucleic acids in the cell, the expression of said gene leading to formation of the nuclease in the cell at a rate which results in the presence of nicks in one strand of the cell nucleic acids to an extent which cannot be repaired by the nucleic acid repair mechanism of the cell, thereby limiting the function of said cell.

3. A cell according to claim 1 wherein the gene whose expression results in the formation of a hydrolytically active enzyme is contained in a recombinant replicon or recombinant replicons.

4. A cell according to claim 3 wherein the regulatory sequence is contained in a recombinant replicon or recombinant replicons.

5. A cell according to claim 3 wherein the gene and the regulatory sequence are contained in the same recombinant replicon.

6. A cell according to claim 5 which contains a multiplicity of replicons each containing both the gene and the regulatory sequence.

7. A cell according to claim 1 wherein the gene whose expression results in the formation of an hydrolytically active enzyme is a gene lacking a sequence coding for a peptide signal sequence which, if present, would have permitted the enzyme to be transported through the cell membrane.

8. A cell according to claim 1 wherein the gene whose expression results in the formation of a truncated and/or mutated *Staphylococcus aureus* nuclease is derived from *Staphylococcus aureus* (Foggi) ATCC 27355.

9. A cell according to claim 8 wherein the expression of the gene results in the formation of a truncated nuclease.

10. A cell according to claim 9 wherein the truncated nuclease lacks at least 9 amino acid residues of those of the parent enzyme.

11. A cell according to claim 8 wherein the nuclease is encoded by the DNA sequence of Fig. 3 (SEQ ID NO:7).

12. A cell according to claim 1 wherein the regulatory nucleotide sequence contains a regulatable promoter operably linked to the gene encoding the hydrolytically active enzyme.

13. A cell according to claim 12 wherein the regulatable promoter is regulated by a factor selected from the environmental conditions of the cell, the physiological state of the cell and a stochastic event.

14. A cell according to claim 13 wherein the regulatable promoter is regulated by a factor selected from the physical conditions in the environment and the presence or absence of a certain chemical in the environment.

15. A cell according to claim 14 wherein the chemical is selected from a carbon source, a nitrogen source, a metabolite, an amino acid, a nucleoside, a pyrimidine base, a

purine base, a metal ion and isopropyl-beta-D-thiogalactopyranoside (IPTG).

16. A cell according to claim 1 wherein the expression of the gene whose expression results in the formation of a hydrolytically active enzyme is stochastically induced as a result of a recombinational excision of an excisable negatively functioning regulatory nucleotide sequence being operably linked to said gene which nucleotide sequence, while present in the cell, inhibits expression of the gene whose expression results in the formation of the hydrolytically active enzyme.

17. A cell according to claim 18 wherein said excisable negatively functioning regulatory nucleotide sequence is a sequence which is flanked by a first flanking sequence and a second flanking sequence substantially homologous with said first flanking sequence whereby said regulatory element is recombinationally excisable in the cell.

18. A cell according to claim 17 wherein the gene whose expression results in the formation of a hydrolytically active enzyme encodes a first RNA which is a messenger RNA, and the excisable negatively functioning regulatory nucleotide sequence being operably linked to said gene, is a gene encoding a second RNA which forms an RNA-RNA duplex with said first messenger RNA and thereby, when it is expressed, inhibits translation of said gene coding for the hydrolytically active enzyme.

19. A cell according to claim 17 wherein the excisable negatively functioning regulatory nucleotide sequence is a gene encoding a polypeptide repressor of transcription of the gene whose expression results in the formation of a hydrolytically active enzyme.

20. A cell according to claim 19 wherein the excisable negatively functioning regulatory nucleotide sequence is a gene encoding a *lac* repressor, and the gene whose expression

results in the formation of a hydrolytically active enzyme is operably linked to the *lac* promoter, said *lac* promoter including the operator site for the *lac* repressor.

21. A cell according to claim 17 wherein the negatively functioning regulatory nucleotide sequence is a termination sequence preventing transcription of the gene whose expression results in the formation of a hydrolytically active enzyme.

22. A cell according to claim 1 wherein the expression of the gene whose expression results in the formation of a hydrolytically active enzyme is stochastically induced as a result of a site-specific recombinational excision of an excisable negatively functioning regulatory nucleotide sequence being operably linked to said gene which nucleotide sequence, while present in the cell, inhibits expression of the gene whose expression results in the formation of the hydrolytically active enzyme.

23. A cell according to claim 22 wherein said excisable negatively functioning regulatory nucleotide sequence is a sequence which is flanked by a first site for site-specific resolution and a second site for site-specific resolution, the second site being resolvable by the same or a functionally equivalent multimer resolving enzyme as is the first site, whereby said regulatory element is recombinationally excisable in the cell.

24. A cell according to claim 22 wherein the first and second site for site-specific resolution is the mrs site derived from plasmid RP4.

25. A cell according to claim 23 wherein the multimer resolving enzyme is encoded by a gene which is located in *trans* relative to the sites for site-specific resolution.

26. A cell according to claim 25 wherein the gene coding for the multimer resolving enzyme is the *parA* gene of plasmid RP4.

27. A cell according to claim 22 wherein the gene whose expression results in the formation of a hydrolytically active enzyme encodes a first messenger RNA, and the excisable negatively functioning regulatory nucleotide sequence being operably linked to said gene, is a gene encoding a second messenger RNA which forms an RNA-RNA duplex with said first messenger RNA and thereby, when it is expressed, inhibits translation of said gene coding for the hydrolytically active enzyme.

28. A cell according to claim 22 wherein the excisable negatively functioning regulatory nucleotide sequence is a gene encoding a polypeptide repressor of transcription of the gene whose expression results in the formation of a hydrolytically active enzyme.

29. A cell according to claim 28 wherein the excisable negatively functioning regulatory nucleotide sequence is a gene encoding a *lac* repressor, and the gene whose expression results in the formation of a hydrolytically active enzyme is operably linked to the *lac* promoter, said *lac* promoter including the operator site for the *lac* repressor.

30. A cell according to claim 22 wherein the negatively functioning regulatory nucleotide sequence is a termination sequence preventing transcription of the gene whose expression results in the formation of a hydrolytically active enzyme.

31. A cell according to claim 1 wherein the gene whose expression results in the formation of a hydrolytically active enzyme is stochastically expressed as a result of recombinational inversion of an invertible promoter sequence of said

regulatory nucleotide sequence said promoter being operably linked to the gene.

32. A cell according to claim 31 wherein the promoter sequence is a sequence carrying the *fimA* promoter or a functional homologue thereof.

33. A cell according to claim 1 wherein at least one of (i) the gene whose expression results in the formation of a hydrolytically active enzyme and (ii) the nucleotide sequence regulating said gene is mutated at one or more sites, whereby the cell function-limiting effect of the enzyme encoded by the gene, when expressed in the cell is the same or increased relative to the cell function-limiting effect of the enzyme expressed in a cell containing said gene and said nucleotide sequence in non-mutated form.

34. A cell according to claim 1 wherein the nucleotide sequence regulating the transcription of the gene whose expression results in the formation of a hydrolytically active enzyme is derived from a replicon selected from the group consisting of a bacterial plasmid, a bacterial chromosome, a prokaryotic virus, a eucaryotic plasmid, a eucaryotic virus, a eucaryotic chromosome, eucaryotic mitochondria, a eucaryotic chloroplast, and a synthetic sequence.

35. A cell according to claim 1 which comprises a further regulatably expressible gene which encodes a further cell function-limiting gene product.

36. A cell according to claim 35 wherein the cell function-limiting gene product is an exonuclease including the gene product encoded by the *E. coli xth* gene.

37. A cell according to claim 35 wherein the further regulatably expressible gene is regulated by a regulatory nucleotide sequence of the same type as the sequence regulating

the gene whose expression results in the formation of a hydrolytically active enzyme.

38. A cell according to claim 35 wherein the further regulatably expressible gene is regulated by a regulatory nucleotide sequence of another type than the sequence regulating the gene whose expression results in the formation of a hydrolytically active enzyme, said other type being a regulatory nucleotide sequence which is also capable of regulating the gene whose expression results in the formation of the hydrolytically active enzyme.

39. A cell according to claim 35 wherein the further regulatably expressible gene is selected from the *hok* gene from the *parB* region of plasmid R1 and a DNA sequence which is functionally homologous to the R1 *hok* gene including the *gef* gene.

40. A cell according to claim 1 comprising a further DNA sequence not naturally related to the replicon carrying the gene whose expression results in the formation of a hydrolytically active enzyme and/or the replicon carrying the regulatory nucleotide sequence, said further DNA sequence being selected from a sequence coding for an immunologically active gene product, a sequence coding for a pesticidally active gene product and a sequence coding for a pollutant-degrading gene product.

42. A cell according to claim 1 which is a cell transformed with a recombinant replicon or recombinant replicons containing a gene whose expression results in the formation of an enzyme which is present and hydrolytically active in the cytoplasm of said cell, the expression of said gene leading to formation of the enzyme in the cell at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cell, to an extent whereby the function of the cell is limited, the expression of said gene being regulated by a regulatory

nucleotide sequence which is contained in the recombinant replicon containing the gene or in another recombinant replicon present in the transformed cell.

43. A recombinant replicon containing a regulatably expressible gene which, when expressed in a cell encodes a truncated and/or mutated *Staphylococcus aureus* nuclease which is present and hydrolytically active in the cytoplasm of the cell, the expression of said gene leading to formation of the enzyme in the cell at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cell, to an extent whereby the function of the cell is being limited, the expression of said genes being regulated by a regulatory nucleotide sequence which is contained in the recombinant replicon or in another recombinant replicon present in a cell containing the replicon.

44. A recombinant replicon according to claim 43 which contains the regulatory nucleotide sequence.

45. A replicon according to claim 1 wherein the gene whose expression results in the formation of a truncated and/or mutated *Staphylococcus aureus* nuclease is derived from *Staphylococcus aureus* (Foggi) ATCC 27355.

46. A replicon according to claim 45 wherein the nuclease lacks at least 9 amino acid residues of those of the parent enzyme.

47. A replicon according to claim 46 wherein the nuclease is encoded by the DNA sequence of Fig. 3 (SEQ ID NO:7).

48. A replicon according to claim 43 which is selected from pSNUC24-26 and pSNUC420-26.

49. A replicon according to claim 43 wherein the expression of the gene results in the formation of a nuclease being capable of hydrolysing diesterphosphate bonds in nucleic acids in the cell, the expression of said gene leading to formation of the nuclease in the cell at a rate which results in the presence of nicks in one strand of the cell nucleic acids to an extent which cannot be repaired by the nucleic acid repair mechanism of the cell, thereby limiting the function of said cell.

50. A replicon according to claim 43 wherein the gene encoding the hydrolytically active enzyme is a gene lacking a sequence coding for a peptide signal sequence which, if present, would have permitted the enzyme to be transported through the cell membrane.

51. A replicon according to claims 43 wherein the regulatory nucleotide sequence contains a regulatable promoter operably linked to the gene encoding the hydrolytically active enzyme.

52. A replicon according to claim 51 wherein the regulatable promoter is regulated by a factor selected from the environmental conditions of a cell containing the replicon, the physiological state of the cell and a stochastic event.

53. A replicon according to claim 52 wherein the regulatable promoter is regulated by the presence or the absence of a chemical in the environment of a cell containing the replicon, said chemical being selected from a carbon source, a nitrogen source, a metabolite, an amino acid, a nucleoside, a pyrimidine base, a purine base, a metal ion and isopropyl-beta-D-thiogalactopyranoside (IPTG).

54. A replicon according to claim 43 wherein the expression of the gene coding for the hydrolytically active enzyme is stochastically induced as a result of a recombinational excision of an excisable negatively functioning regulatory

nucleotide sequence which, while present in the cell, inhibits expression of the gene coding for the hydrolytically active enzyme.

55. A replicon according to claim 54 wherein said excisable negatively functioning regulatory nucleotide sequence is a sequence flanked by a first flanking sequence and a second flanking sequence substantially homologous with said first flanking sequence whereby said regulatory element is recombinationally excisable in the cell.

56. A replicon according to claim 54 wherein the gene coding for the hydrolytically active enzyme encodes a first RNA which is a messenger RNA, and the excisable negatively functioning regulatory nucleotide sequence is a gene encoding a second RNA which forms an RNA-RNA duplex with said first messenger RNA and thereby inhibits translation thereof.

57. A replicon according to claim 54 wherein the negatively functioning regulatory nucleotide sequence is a gene encoding a polypeptide repressor of transcription of the gene coding for the hydrolytically active enzyme.

58. A replicon according to claim 57 wherein the negatively functioning regulatory nucleotide sequence is a gene encoding the *lac* repressor, and the gene coding for the hydrolytically active enzyme is operably linked to the *lac* promoter, said *lac* promoter including the operator site for said *lac* repressor.

59. A replicon according to claim 54 wherein the negatively functioning regulatory nucleotide sequence is a termination sequence preventing transcription of the gene coding for the hydrolytically active enzyme.

60. A replicon according to claim 43 wherein the expression of the gene whose expression results in the formation of a hydrolytically active enzyme is stochastically induced as a

result of a site-specific recombinational excision of an excisable negatively functioning regulatory nucleotide sequence being operably linked to said gene which nucleotide sequence, while present in the cell, inhibits expression of the gene whose expression results in the formation of the hydrolytically active enzyme.

61. A replicon according to claim 60 wherein said excisable negatively functioning regulatory nucleotide sequence is a sequence which is flanked by a first site for site-specific resolution and a second site for site-specific resolution, the second site being resolvable by the same or a functionally equivalent multimer resolving enzyme as is the first site, whereby said regulatory element is recombinationally excisable in the cell.

62. A replicon according to claim 60 wherein the first and second site for site-specific resolution is the mrs site derived from plasmid RP4.

63. A replicon according to claim 61 wherein the multimer resolving enzyme is encoded by a gene which is located in *trans* relative to the sites for site-specific resolution.

64. A replicon according to claim 63 wherein the gene coding for the multimer resolving enzyme is the *parA* gene of plasmid RP4.

65. A replicon according to claim 60 wherein the gene whose expression results in the formation of a hydrolytically active enzyme encodes a first RNA which is a messenger RNA, and the excisable negatively functioning regulatory nucleotide sequence being operably linked to said gene, is a gene encoding a second RNA which forms an RNA-RNA duplex with said first messenger RNA and thereby, when it is expressed, inhibits translation of said gene coding for the hydrolytically active enzyme.

66. A replicon according to claim 60 wherein the excisable negatively functioning regulatory nucleotide sequence is a gene encoding a polypeptide repressor of transcription of the gene whose expression results in the formation of a hydrolytically active enzyme.

67. A replicon according to claim 66 wherein the excisable negatively functioning regulatory nucleotide sequence is a gene encoding a *lac* repressor, and the gene whose expression results in the formation of a hydrolytically active enzyme is operably linked to the *lac* promoter, said *lac* promoter including the operator site for the *lac* repressor.

68. A replicon according to claim 60 wherein the negatively functioning regulatory nucleotide sequence is a termination sequence preventing transcription of the gene whose expression results in the formation of a hydrolytically active enzyme.

69. A replicon according to claim 43 wherein the gene coding for the hydrolytically active enzyme is stochastically expressed as a result of recombinational inversion of an invertible promoter sequence of said regulatory nucleotide sequence said promoter being operably linked to the gene coding for the enzyme.

70. A replicon according to claim 69 wherein the promoter sequence is a sequence carrying the *fimA* promoter or a functional homologue thereof.

71. A replicon according to claim 43 wherein the gene coding for the hydrolytically active enzyme or the nucleotide sequence regulating said gene is mutated at one or more sites, whereby the cell function-limiting effect of the enzyme encoded by the gene, when expressed in the cell is the same or increased relative to the cell function-limiting effect of the enzyme expressed in a cell not containing mutated DNA.

72. A replicon according to claim 71 wherein the gene product of the mutated gene is an enzyme having an enzymatic activity which is at least about 2-fold of that of the wildtype enzyme.

73. A replicon according to claim 43 which is a replicon comprising a further regulatably expressible gene encoding a cell function-limiting function.

74. A replicon according to claim 73 wherein the further regulatably expressible gene is regulated by a regulatory nucleotide sequence of the same type as the sequence regulating the gene coding for the cell function-limiting hydrolytically active enzyme.

75. A replicon according to claim 73 wherein the further regulatably expressible gene is selected from the *hok* gene from the *parB* region of plasmid R1 and a DNA sequence which is functionally homologous to the R1 *hok* gene including the *gef* gene.

76. A replicon according to claim 73 wherein the cell function-limiting gene product is an exonuclease including the gene product encoded by the *E. coli xth* gene.

77. A replicon according to claim 50 which is a replicon comprising a further DNA sequence not naturally related to the replicon carrying the gene coding for the hydrolytically active enzyme and/or the replicon carrying the regulatory nucleotide sequence, said further DNA sequence being selected from a sequence coding for an immunologically active gene product, a sequence coding for a pesticidally active gene product and a sequence coding for a pollutant-degrading gene product.

78. A population of cells consisting of a multiplicity of cells as defined in claim 1.

79. A cell population according to claim 78 comprising transformable cells having been transformed with the recombinant replicon as defined in claim 43, said replicon being capable of replicating in said cells.

80. A cell population according to claim 78 wherein the cells are bacterial cells.

81. A cell population according to claim 80 wherein the bacterial cells are selected from species whose natural habitat is a habitat selected from soil, surface water and plants.

82. A cell population according to claim 80 wherein the bacterial cells are gram-negative bacterial cells.

83. A cell population according to claim 80 wherein the expression of the gene coding for the function-limiting enzyme is regulatable by a repressor substance which can undergo a decay when said cells are released to a human or animal body or the outer environment to an extent whereby the repressor substance is converted to a non-functional form, said repressor substance being present in the cells of the population in different amounts whereby as a result of said linear decay, the function of the cells of the population will be gradually limited.

84. A method of limiting the survival of a cell population in a first or a second environment which method comprises transforming the cells of said population with a recombinant replicon being replicated in the cells of the population and containing a gene whose expression results in the formation of a truncated and/or mutated *Staphylococcus aureus* nuclease which is present and hydrolytically active in the cytoplasm of said cell, the cells further containing a regulatory nucleotide sequence being regulatable by a factor in said first or second environment, the presence or absence of which regulates the expression of the gene, the expression of said

gene leading to formation of the enzyme in the cells at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cell, to an extent whereby the function of the cells is being limited leading to a limitation of the survival of the cell population.

85. A method according to claim 84 wherein the cell population is the cell population as defined in claims 78-83.

86. A method according to claim 84 wherein the survival of the cell population is limited in a first environment in which the gene is expressed, said cell population thereby being contained in said first environment.

87. A method according to claim 84 wherein the survival of the cell population is not limited when present in a first environment, which first environment could change to a second environment physically and/or chemically distinct from the first environment, in which first environment the gene whose expression results in the formation of a hydrolytically active enzyme is not expressed, but the survival of which cell population is limited when transferred to a second environment or when present in a physically and/or chemically changed first environment, where the gene is expressed.

88. A method according to claim 84 wherein the survival of a cell population is being limited by providing in the cells a gene coding for the hydrolytically active enzyme which is operably linked to a regulatory nucleotide sequence encoding a repressor substance which can undergo a decay when said cells are released to a human or animal body or the outer environment to an extent whereby the repressor substance is converted to a non-functional form, said repressor substance being present in the cells of the population in different amounts whereby as a result of said decay, the function of the cells of the population will be gradually be limited.

89. A method of containing an extrachromosomal recombinant replicon to a first kind of cell, where said replicon is naturally transferable to a second kind of cell, which method comprises providing on the recombinant extrachromosomal replicon a gene whose expression results in the formation of a truncated and/or mutated *Staphylococcus aureus* nuclease which is present and hydrolytically active in the cytoplasm of a cell, the formation of said enzyme being at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cell, to an extent whereby the function of the cell is being limited, said first kind of cells having or being modified to have a chromosomal replicon comprising a regulatory nucleotide sequence which inhibits the expression of said gene and thereby protects said first kind of cells, said regulatory gene being lacking in said second kind of cell, whereby, if a cell of the second kind receives said extrachromosomal recombinant replicon said gene is expressed and has a function-limiting effect thereon.

90. A method according to claim 89 wherein the recombinant replicon is a replicon as defined in claim 43 which does not contain a regulatory nucleotide sequence.

91. A method according to claim 89 wherein the expression of the gene whose expression results in the formation of a hydrolytically active enzyme, results in the formation of an endonuclease being capable of hydrolysing diesterphosphate bonds in nucleic acids in the cell, the expression of said gene leading to formation of the endonuclease in the cell at a rate which results in the presence of nicks in the cell nucleic acids to an extent which cannot be repaired by the nucleic acid repair mechanism of the cells, thereby limiting the function of said cell.

92. A method of stochastically limiting the survival of a cell population which comprises transforming the cells thereof with a recombinant replicon containing a regulatably

expressible gene which, when expressed in a cell encodes a truncated and/or mutated *Staphylococcus aureus* nuclease which is present and hydrolytically active in the cytoplasm of the cell, the expression of said gene leading to formation of the enzyme in the cell at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cells, to an extent whereby the function of the cells is being limited, the expression of said genes or genes being stochastically induced as a result of recombinational excision of an excisable negatively functioning regulatory nucleotide sequence which, while present in the cells, inhibits expression of the gene coding for the enzyme, said negatively functioning regulatory nucleotide sequence being contained in the recombinant replicon or in an other recombinant replicon present in cells of the population containing the replicon.

93. A method according to claim 92 wherein the recombinant replicon is the replicon as defined in claim 54.

94. A method of stochastically limiting the survival of a cell population, comprising transforming the cells thereof with a recombinant replicon containing a regulatably expressible gene which, when expressed in a cell encodes a truncated and/or mutated *Staphylococcus aureus* nuclease which is present and hydrolytically active in the cytoplasm of the cell, the expression of said gene leading to formation of the enzyme in the cell at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cells, to an extent whereby the function of the cells is being limited, the expression of said gene being stochastically induced as a result of a site-specific recombinational excision of an excisable negatively functioning regulatory nucleotide sequence which, while present in the cells, inhibits expression of the gene coding for the enzyme, said negatively functioning regulatory nucleotide sequence being contained in the recombinant repli-

con or in an other recombinant replicon present in cells of the population containing the replicon.

95. A method according to claim 94 wherein the recombinant replicon is the replicon as defined in claim 73.

96. A method of stochastically limiting the survival of a cell population which comprises transforming the cells thereof with a recombinant replicon containing a regulatably expressible gene which, when expressed in the cells of the population encodes a truncated and/or mutated *Staphylococcus aureus* nuclease not being transportable over the cell membrane of said cells, and which is hydrolytically active in the cytoplasm of the cells, the expression of said gene leading to formation of the enzyme in the cells at a rate which results in the hydrolysis of hydrolysable cytoplasmic substances necessary for non-limited function of the cells, to an extent whereby the function of the cells is being limited, the expression of said gene being stochastically expressed as a result of recombinational inversion of an invertible promoter sequence of said regulatory nucleotide sequence, said promoter being operably linked to the gene coding for the enzyme which, while present in the cells, inhibits expression of the gene coding for the enzyme, said regulatory nucleotide sequence being contained in the recombinant replicon or in an other recombinant replicon present in a cells containing the replicon.

97. A method according to claim 96 wherein the promoter sequence is a sequence carrying the *fimA* promoter or functional homologue thereof.

98. An immunologically active composition which contains a viable function-limited cell population as defined in claim 78 wherein the cells contain a further DNA sequence not naturally related to the gene coding for the hydrolytically active enzyme or to the regulatory nucleotide sequence, which further sequence is a sequence coding for an immunologically

active gene product, the cells being function-limited to an extent which, when the composition is administered to a human or an animal, allows the cells to express the immunologically active gene product for a period of time and in an amount sufficient to obtain an effective immune response in said human or animal, but which does not allow the cells to persist in the human or the animal.

99. A composition according to claim 98 wherein the cells therein contain a sequence coding for an immunologically active gene product which is a sequence coding for a fusion protein comprising the immunologically active gene product and a polypeptide, the presence of which results in the transportation of said fusion protein to the outer surface of the cells.

100. A composition according to claim 99 wherein the polypeptide being present in the fusion protein is a cell surface polypeptide selected from a polypeptide derived from fimbrillin protein, a pilus, a flagellum, an OM surface protein.

101. A composition according to claim 99 wherein the cell surface polypeptide is derived from a bacterial species selected from *Enterobacteriaceae*, *Vibrionaceae* and *Pseudomonadaceae*.

102. A pesticidally active composition which contains a viable cell population as defined in claim 78 wherein the cells contain a further DNA sequence not naturally related to the gene coding for the hydrolytically active enzyme or to the regulatory nucleotide sequence, which further sequence is a sequence coding for a pesticidally active gene product, the cells being function-limited to an extent which, when the composition is administered to an environment containing a pest, allows the cells to express the pesticidally active gene product for a period of time and in an amount sufficient to obtain an effective pesticidal effect in said environment

but which does not allow the cells to persist in the environment.

103. A composition according to claim 102 wherein the further DNA sequence codes for a gene product which is toxic for insects or their progeny.

104. A composition according to claim 103 wherein the further DNA sequence is a sequence derived from a strain of *Bacillus thuringiensis* encoding an insecticidal protein.

105. An environmental pollutant-degrading composition which contains a viable cell population as defined in claim 78 wherein the cells contains a further DNA sequence not naturally related to the gene coding for the hydrolytically active enzyme or to the regulatory nucleotide sequence, which further sequence is a sequence coding for an environmental pollutant-degrading gene product, the cells being function-limited to an extent which, when the composition is administered to an environment containing a pollutant to be degraded, allows the cells to express said pollutant degrading gene product for a period of time and in an amount sufficient to obtain an effective pollutant-degrading effect in said environment but which does not allow the cells to persist in the environment.

106. A composition according to claim 105 wherein the gene or genes the expression of which results in the formation of a cell function-limiting enzyme is only expressed when the pollutant degradable by the pollutant-degrading gene product is substantially degraded.

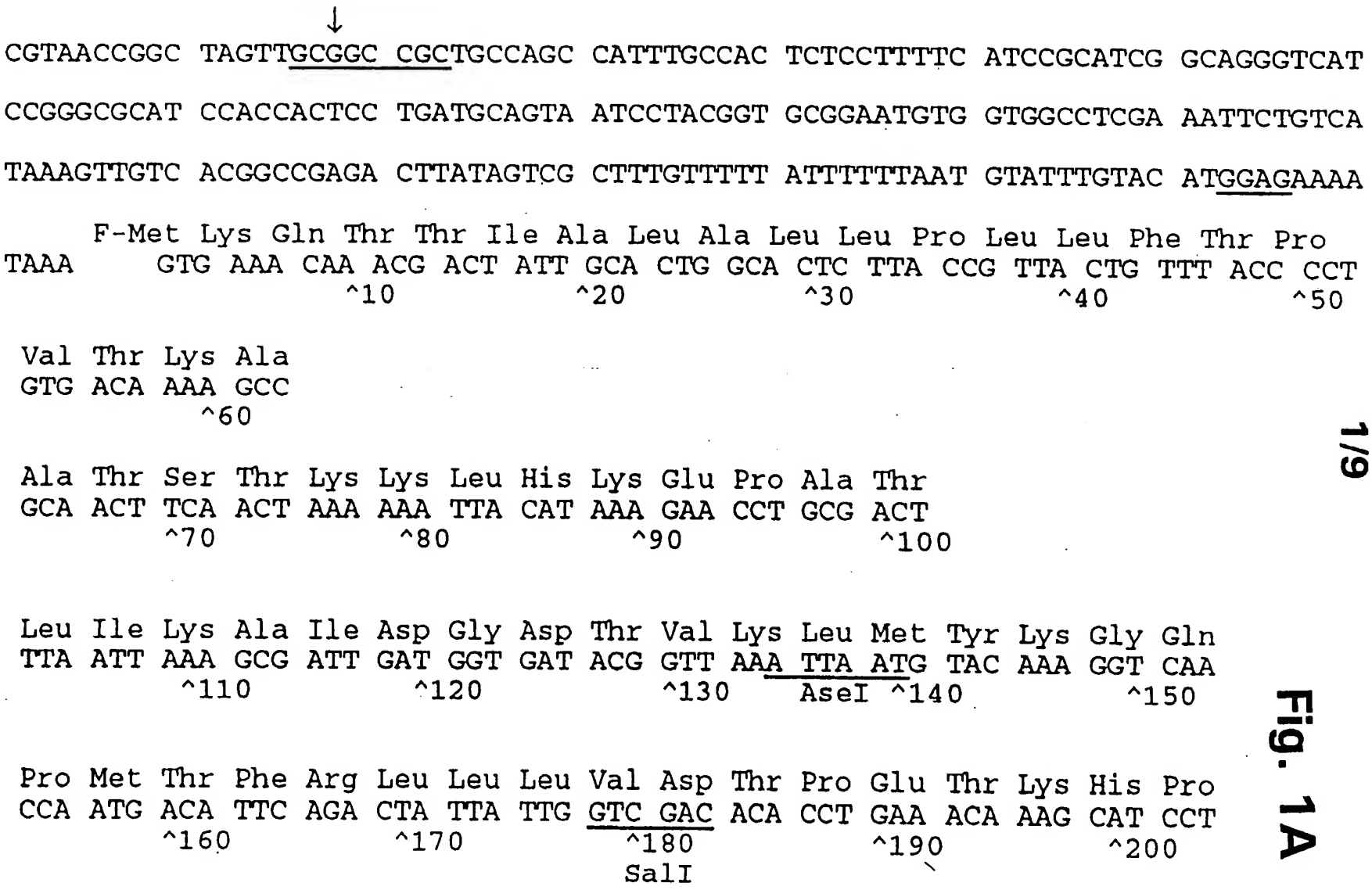

Fig. 1A

```
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      ^210          ^220          ^230          ^240          ^250 Bst XI

Met Val Glu Asn Ala Lys Lys Ile Glu Val Glu Phe Asp Lys Gly Gln Arg
ATG GTA GAA AAT GCA AAG AAA ATT GAA GTC GAA TTC GAC AAA GGT CAA AGA
     ^260          ^270          ^280          ^290          ^300
                                              EcoRI

Thr Asp Lys Tyr Gly Arg Gly Leu Ala Tyr Ile Tyr Ala Asp Gly Lys Met
ACT GAT AAA TAT GGA CGT GGG CTA GCG TAT ATT TAT GCT GAT GGA AAA ATG
    ^310          ^320          ^330          ^340          ^350
                                NheI

Val Asn Glu Ala Leu Val Arg Gln Gly Leu Ala Lys Val Ala Tyr Val Tyr
GTA AAC GAA GCT TTA GTT CGT CAA GGC TTG GCT AAA GTT GCT TAT GTT TAC
  ^360          ^370          ^380          ^390          ^400
          HindIII

Lys Pro Asn Asn Thr His Glu Gln His Leu Arg Lys Ser Glu Ala Gln Ala
AAA CCT AAC AAT ACA CAT GAA CAA CAT TTA AGA AAA AGT GAA GCA CAA GCG
 ^410          ^420          ^430          ^440          ^450

Lys Lys Glu Lys Leu Asn Ile Trp Ser Glu Asp Asn Ala Asp Ser Gly Gln
AAA AAA GAG AAA TTA AAT ATT TGG AGC GAA GAC AAC GCT GAT TCA GGT CAA
^460          ^470          ^480          ^490          ^500
                      SspI

ter
TAA TGCTCATTGT AAAAGTGTCA CTGCTGCTAG TGGCAC
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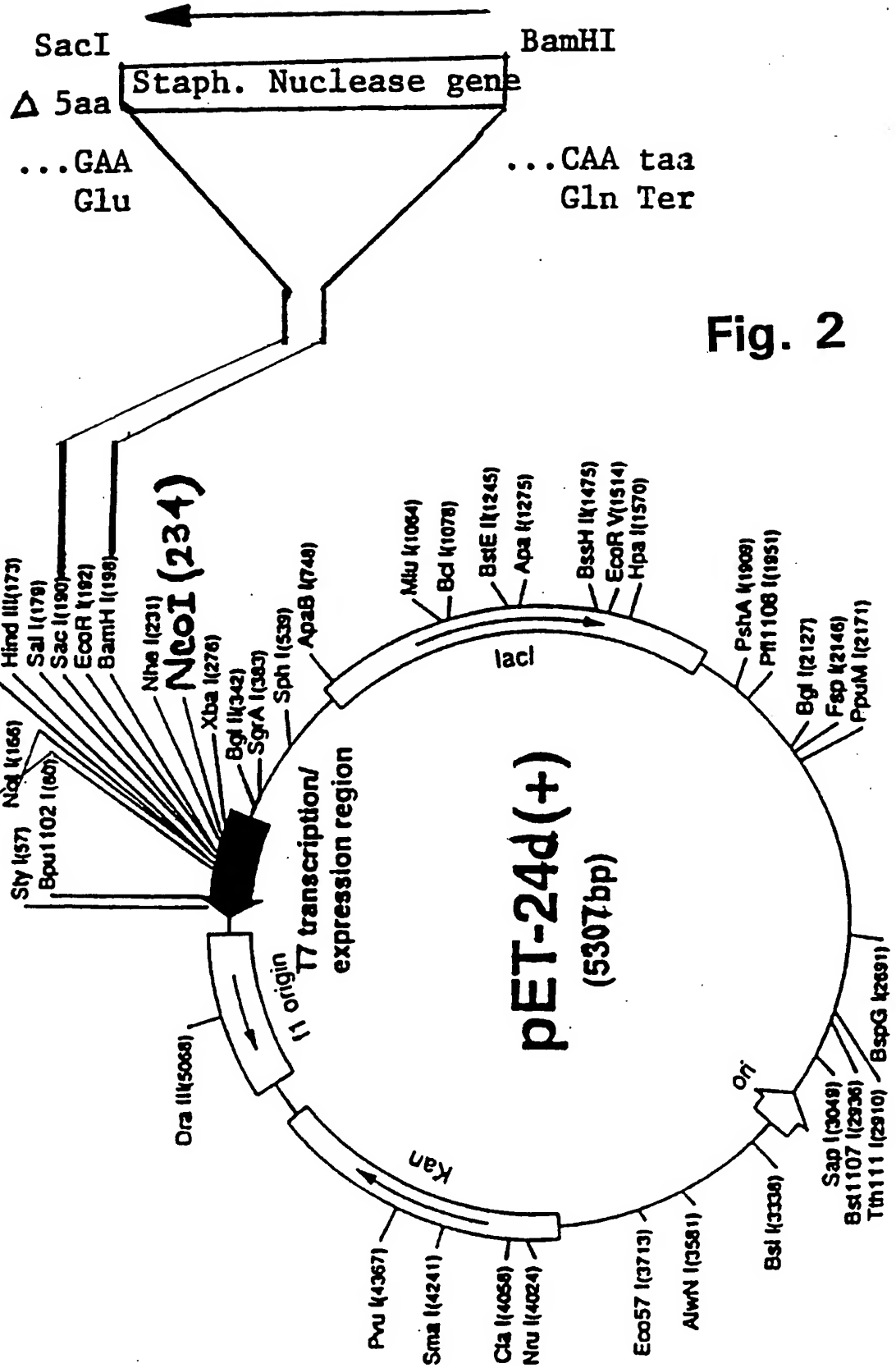
Fig. 1B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BamHI
Staph. Nuclease gene
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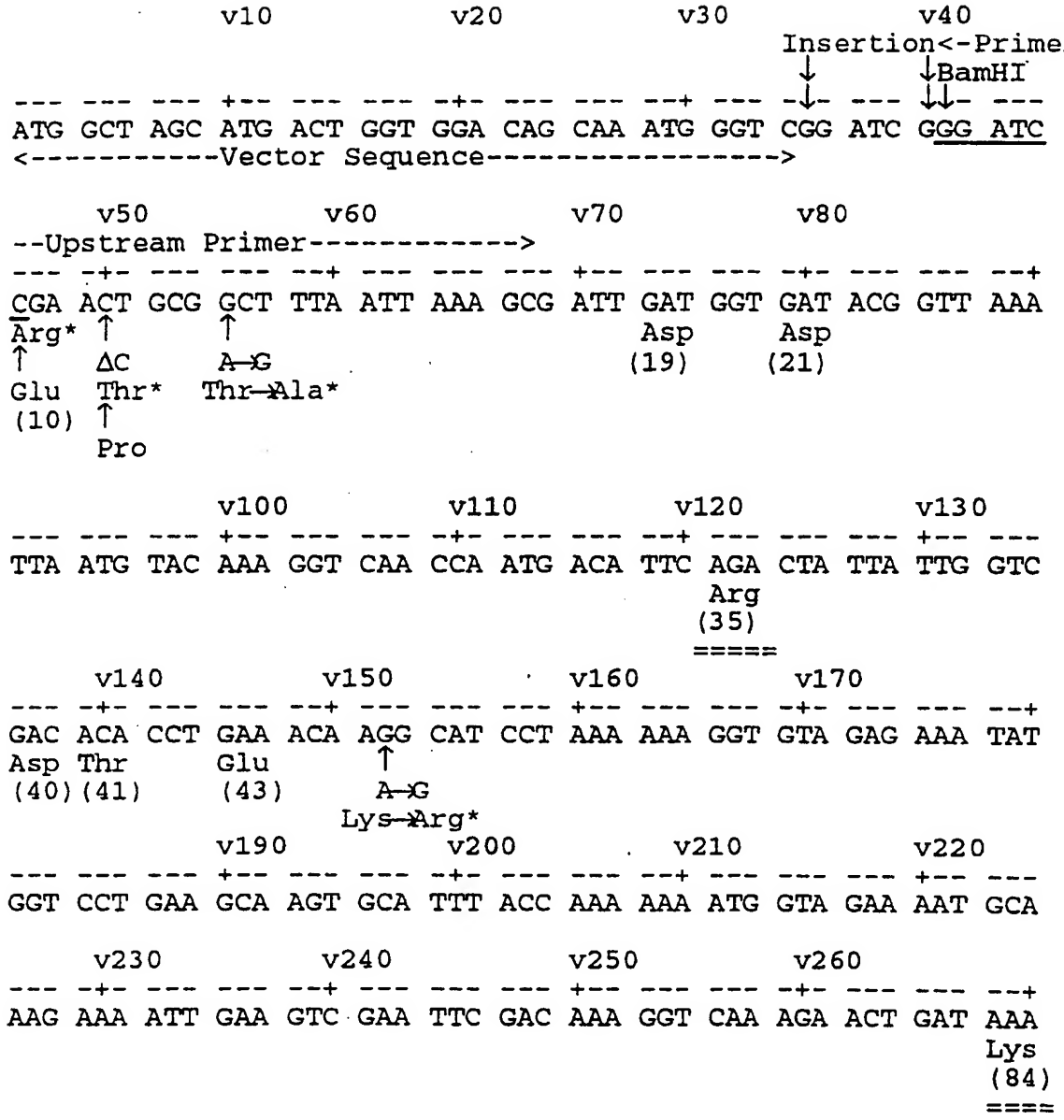
...GAA
Glu
...CAA taa
Gln Ter
NcoI (234)
pET-24d(+)
(5307bp)
T7 transcription/
expression region
lacI
Kan
ori
f1 origin

Fig. 2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BamHI
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v50 v60 v70 v80
--Upstream Primer---------->
CGA ACT GCG GCT TTA ATT AAA GCG ATT GAT GGT GAT ACG GTT AAA
Arg* ΔC A→G Asp Asp
Glu Thr* Thr→Ala* (19) (21)
(10) Pro
v100 v110 v120 v130
TTA ATG TAC AAA GGT CAA CCA ATG ACA TTC AGA CTA TTA TTG GTC
Arg
(35)
v140 v150 v160 v170
GAC ACA CCT GAA ACA AGG CAT CCT AAA AAA GGT GTA GAG AAA TAT
Asp Thr Glu A→G
(40) (41) (43) Lys→Arg*
v190 v200 v210 v220
GGT CCT GAA GCA AGT GCA TTT ACC AAA AAA ATG GTA GAA AAT GCA
v230 v240 v250 v260
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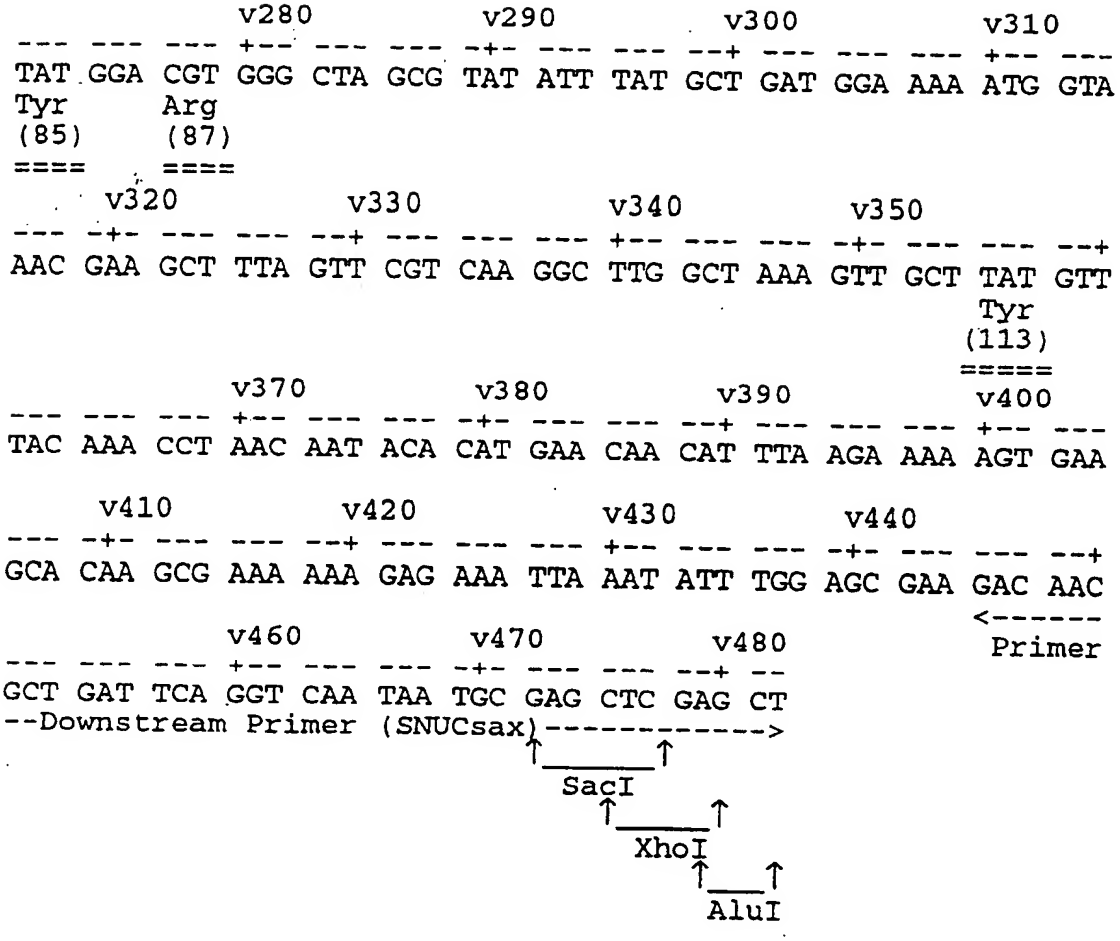
Lys
(84)

Fig. 3A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v290
v300
v310
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Tyr Arg
(85) (87)
v320
v330
v340
v350
AAC GAA GCT TTA GTT CGT CAA GGC TTG GCT AAA GTT GCT TAT GTT
Tyr
(113)
v370
v380
v390
v400
TAC AAA CCT AAC AAT ACA CAT GAA CAA CAT TTA AGA AAA AGT GAA
v410
v420
v430
v440
GCA CAA GCG AAA AAA GAG AAA TTA AAT ATT TGG AGC GAA GAC AAC
Primer
v460
v470
v480
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--Downstream Primer (SNUCsax)
SacI
XhoI
AluI

Fig. 3B

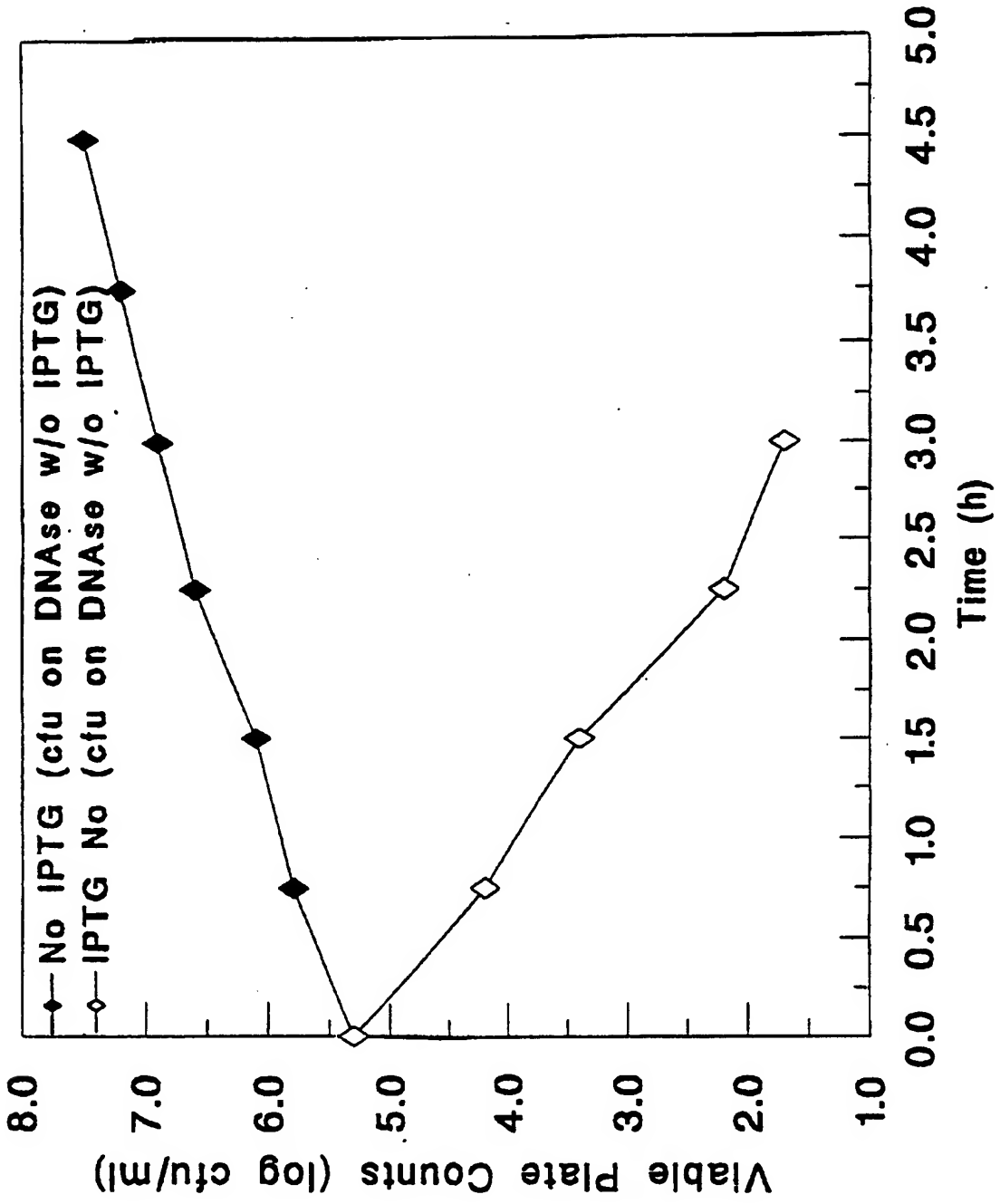
No IPTG (cfu on DNAse w/o IPTG)
IPTG No (cfu on DNAse w/o IPTG)
Viable Plate Counts (log cfu/ml)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Time (h)

Fig. 4

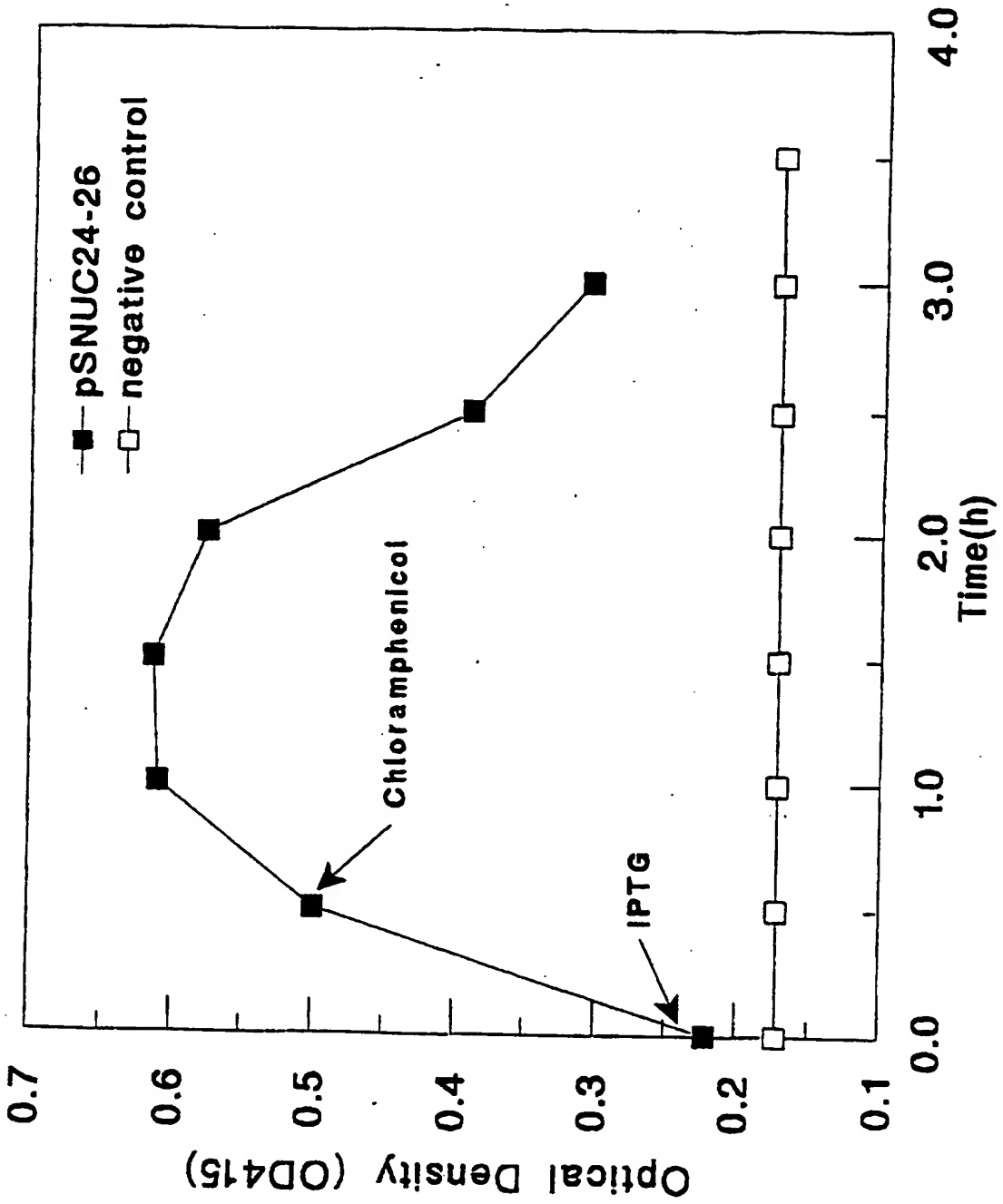
pSNUC24-26
negative control
Chloramphenicol
IPTG
Optical Density (OD415)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1.0
2.0
3.0
4.0
Time(h)

Fig. 5

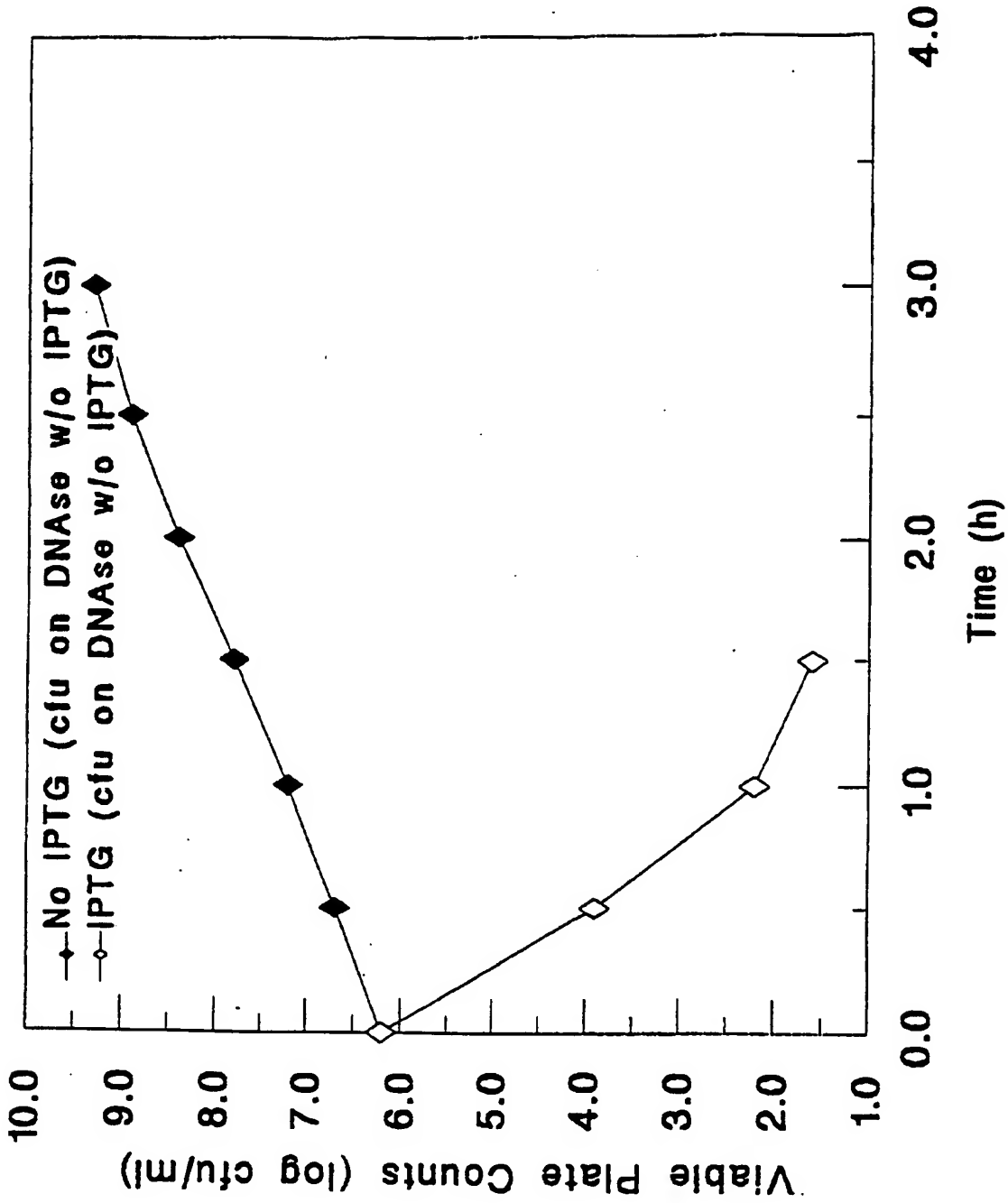
No IPTG (cfu on DNAse w/o IPTG)
IPTG (cfu on DNAse w/o IPTG)
Viable Plate Counts (log cfu/ml)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1.0
2.0
3.0
4.0
Time (h)

Fig. 6

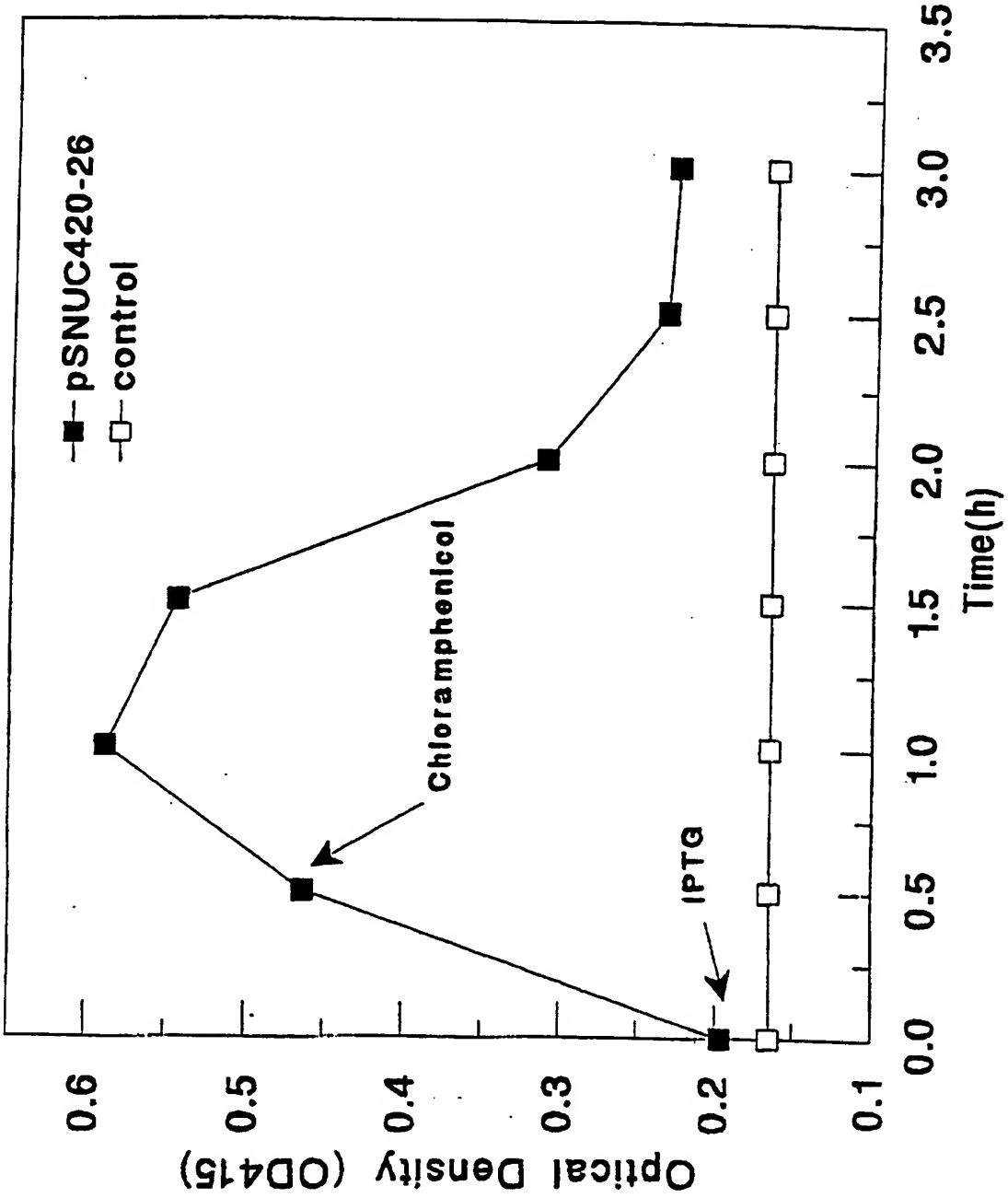
pSNUC420-26
control
Chloramphenicol
IPTG
Optical Density (OD415)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Time(h)

Fig. 7

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/DK 94/00381

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/55 C12N15/32 C12N9/22 C12N15/00 C12N1/21
C12N15/70 C12N15/62 C12S1/00 A61K39/02 A01N63/00
//C07K14/325,(C12N1/21,C12R1:19)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,93 20211 (PLOUGMANN & VINGTHOFT A/S) 14 October 1993 cited in the application see the whole document especially page 61 lines 19-30 and page 62 ---	1-106
A	J. BACTERIOL., vol.164, no.2, November 1985 pages 925 - 928 LISS ET AL. 'Export defect adjacent to the processing site of Staphylococcal nuclease is suppressed by a prlA mutation' see the whole document ---	1-106
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 December 1994	23 -01- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gac, G

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/DK 94/00381

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GENETICS, vol.110, no.4, August 1985 pages 539 - 555 SHORTLE ET AL. 'Genetic analysis of Staphylococcal nuclease : identification of three intragenic "global" suppressors of nuclease-minus mutations' see whole document, especially pages 547 and 548 ---	1-106
A	WO,A,88 09821 (MASSACHUSETS INSTITUTE OF TECHNOLOGY) 15 December 1988 see page 5, line 17 - line 21 see page 6, line 5 - line 6 see page 8, line 4 - line 22 see page 10, line 20, paragraph 26 see page 11, line 9 ---	1-106
A	WO,A,87 05932 (PLOUGHMANN & VINGTOFT) 8 October 1987 -----	1-106

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INTERNATIONAL SEARCH REPORT

..formation on patent family members

Inter nal Application No
PCT/DK 94/00381

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		CA-A-	2133345	14-10-93
WO-A-8809821	15-12-88	US-A-	4965197	23-10-90
WO-A-8705932	08-10-87	AT-T-	107697	15-07-94
		AU-A-	7232087	20-10-87
		AU-B-	646467	24-02-94
		AU-A-	7818391	14-11-91
		DE-D-	3750125	28-07-94
		DE-T-	3750125	27-10-94
		EP-A-	0273040	06-07-88
		JP-T-	1500002	12-01-89